



# Ecological Patterns and Processes in Sarracenia Carnivorous Pitcher Plant Fungi

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**Ecological patterns and processes in *Sarracenia* carnivorous pitcher plant fungi**

**ABSTRACT**

The kingdom Fungi is taxonomically and ecologically diverse, containing an estimated 1.5 million species. Fungi include decomposers, pathogens, and plant and animal mutualists. Many fungi are microorganisms, and the processes shaping microbial diversity may be fundamentally different from those that shape plants and animals. However, ecologists do not yet fully understand how fungal species are distributed over space and time. Using fungi that inhabit the water of *Sarracenia* carnivorous pitchers, I describe inter and intraspecific fungal diversity and investigate the processes that shape fungal diversity. I introduce these concepts in Chapter 1.

In Chapter 2, I describe changes in fungal species diversity over space and time. I enumerated fungal species in five *Sarracenia* populations across the United States and Canada, and show that thousands, but not hundreds of kilometers separate distinct fungal communities. I also sampled a single *Sarracenia* population over a *Sarracenia* growing season, and found that young fungal communities are significantly different from older fungal communities. Observed patterns correlate with environmental factors including temperature and pitcher pH, and with the presence or population structure of pitcher inhabiting arthropods.

In Chapter 3, I describe dispersal of and competition among three common pitcher fungi. I tracked *Candida glabrosa*, *Rhodotorula glutinis*, and *Pseudozyma aphidis* appearances in pitchers in a single *Sarracenia* population, and show that different

appearances reflect different dispersal times. I also describe interactions between dispersal and competition in microcosms: high numbers of propagules introduced into a microcosm give a competitive advantage to investigated fungi.

In Chapter 4, I describe changes in genotype composition of a population of *Candida glabrosa*, which is widespread and abundant in pitchers, and disperses early in the season. I observed three *C. glabrosa* populations in five locations; *C. glabrosa* population structure does not reflect broader community structure as described in Chapter 2. Population structure instead correlates with host taxonomy, and I contrast inter and intraspecific diversity patterns and the processes that potentially cause such patterns.



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## **CHAPTER 1**

### **Introduction and motivation**

## Motivation

Ecologists study the distributions of biodiversity, the processes that maintain biodiversity, and biodiversity's functions in nature. In this thesis, I define biodiversity as the identities and distributions of species and genotypes at a particular location, and over space and time. Biodiversity can be organized into communities and populations. I define a community as in Hubbell (2005): “[a] co-occurring assemblage...of trophically similar species.” I define a population as a group of individuals of the same species that recombines freely; populations can be composed of individuals with identical or different genotypes.

We know little about microbial diversity in nature. For example, mycologists have described ~97 000 fungal species (Kirk *et al.* 2008), but estimate that there are 1.5 to 5.1 million fungal species on Earth (Hawksworth 1991, O'Brien *et al.* 2005). Fungal diversity estimates are based on ratios of fungal to plant species in well studied systems. We know even less about bacterial diversity: bacteriologists have described ~5000 bacterial species (Garrrity & Holt 2001). Estimates of numbers of bacterial species on Earth vary by orders of magnitude from 10 000 to 1 trillion (Mora *et al.* 2011, Dykhuizen 1998). Current taxonomic information is not sufficient to precisely estimate the order of magnitude of the number of bacterial taxa on Earth (Pedrós-Alió 2012). In contrast, ~220 000 of the estimated ~300 000 land plant species have been described; this estimate of land plant species diversity is the result of extrapolating species numbers from numbers of higher taxonomic levels (Mora *et al.* 2011).

Humans depend on microbes for survival, and changes in microbial diversity can lead to environmental problems. Microorganisms are responsible for nutrient cycling

(*e.g.*, Nitrogen-cycling bacteria), primary production (*e.g.*, cyanobacteria), decomposition (*e.g.*, wood-decomposing fungi), and regulation of larger organisms through symbioses (*e.g.*, human gut microbes, Madigan *et al.* 2009). All of these are examples of ecological functions: activities of an organism that alter the ecosystem it inhabits. Functions include metabolism and interactions with other organisms. Changes in biodiversity can result in changes in ecological function. For example, intermediate (21-40%) decreases in local biodiversity reduce primary production (by 5-10%), and decreases in litter consumer diversity reduce decomposition by ~8% (Hooper *et al.* 2012). Changes in microbial diversity can also lead to diverse environmental problems including animal diseases (*e.g.*, chytridiomycosis, Berger *et al.* 1998), plant diseases (*e.g.*, sudden oak death, Meentemeyer 2004), and harmful algal blooms (Hallegraeff 1993).

### **Ecological processes**

Competition, environmental filtering, and dispersal are all examples of processes that act on biodiversity in nature, or ecological processes. Ecological processes can increase, decrease, or maintain biodiversity, and they can operate over time, space, or both. They often interact with each other; two ecological processes working in opposition can maintain an equilibrium number of species. An ecological processes can be deterministic if it acts on any particular species or genotype more than others, or stochastic if it acts equally on all species in a community or genotypes in a population. If ecological processes are deterministic in a community or population, then an observer can predict species composition; if ecological processes are stochastic, then the observer cannot predict species composition, but often can predict other qualities of the

community or population, including species number and distribution of abundant or rare species (Hubbell 2001).

For example, competition can interact with environmental filtering to maintain species diversity. Two organisms compete when the presence of one decreases the other's survival, growth, or reproduction (Begon *et al.* 2006). Competition can be direct or indirect. Direct (interference) competition is the result of physical or chemical contact among organisms; for example, wood-decomposing fungi produce physical barriers or toxic chemicals that prevent competing fungi from accessing resources (Boddy 2000). Indirect (exploitation) competition occurs when an organism reduces the availability of a resource (for example, a fungus might reduce the concentration of sugars on a substrate) to a level that is too low to support its competitors. Environmental filtering occurs when environmental conditions do not permit an organism to persist in a location. This definition of environmental filtering is the same as the definition of environmental constraints according to Belyea & Lancaster (1999). Environmental filtering may be absolute: for example, no obligately anoxic bacterium can survive in the presence of oxygen. Environmental filtering may also interact with competition when local environmental conditions allow one organism to outcompete another.

In 1957, Hutchinson formalized the niche concept of ecology, which invokes both competition and environmental filtering. Under the niche concept, each member of a community can only survive under a restricted set of environmental conditions, or niche. Each species in a community has a unique niche, although subsets of multiple species' niches may overlap. Competition always drives all competitors in a particular location except one extinct, given enough time, because exactly one member of any community is



better than all others at competing under a given set of environmental conditions. In the niche ecology framework, environmental heterogeneity permits coexistence of different species in close proximity. The niche concept is a deterministic framework for explaining species diversity.

In addition to competition and environmental filtering, dispersal and dispersal limitation influence diversity in both patchy and continuous systems. Dispersal is the movement of propagules of an organism from one habitat to another; dispersal rate or dispersal ability can be the same among all members of a community or population, or they can vary. An organism experiences dispersal limitation if it cannot arrive at a given location at a given time. This definition of dispersal limitation contrasts with other definitions that include the ability of a propagule to reproduce once it arrives at a location (*e.g.*, Hanson *et al.* 2012). Dispersal can also be saturating: under saturating dispersal all organisms in a community can reach all locations as at all times. Dispersal saturation is an extreme case of no dispersal limitation. Organisms can experience more or less dispersal limitation relative to one another. For example, the rate of dispersal to a given location can vary among organisms in a community, or the timing of dispersal can vary so that some organisms arrive in a habitat patch earlier than others. Dispersal limitation can be caused by a variety of events: a population can have low rates of propagule production, propagules may not survive between habitat patches, or small population sizes may produce limited numbers of propagules.

Dispersal limitation is a key process in the theory of island biogeography (MacArthur & Wilson 1967). The theory of island biogeography invokes dispersal (“immigration” in MacArthur & Wilson 1967) and extinction rates to explain numbers of

species on islands. These authors explained observations of increasing species diversity with increasing island area and decreasing distance to the mainland in many communities including amphibians and reptiles in the West Indies, and birds in the Philippines and New Guinea. They theorized that the rate of dispersal of all organisms to an island is a function of the distance from the island to the mainland, and that the rate of extinction of all organisms from an island is a function of the island's area. Number of species on an island is the species number at which dispersal and extinction rates are at equilibrium. Since MacArthur and Wilson described the theory of island biogeography, ecologists have observed island biogeographic patterns in microbes. Microbial island biogeographic patterns include increasing numbers of bacterial taxa with increasing tree hole volume (Bell *et al.* 2005); increasing numbers of bacterial taxa with increasing carnivorous plant pitcher volume (Peterson *et al.* 2008); increasing numbers of ectomycorrhizal fungal species with increasing tree island area (Peay *et al.* 2007); and decreasing numbers of ectomycorrhizal fungal species with increasing distance between tree islands and the forest mainland (Peay *et al.* 2010).

The theory of island biogeography is a stochastic theory: immigration and extinction rates are rates for the entire community, and the theory does not distinguish rates among species within a community. The neutral theory of ecology extends ideas from the theory of island biogeography to mainland systems (Hubbell 2001). The neutral theory invokes stochastic births, deaths, speciation, and dispersal to explain community patterns, and assumes that rates of all processes are equivalent among members of the community.

## **Metacommunity theory**

Dispersal, competition, and environmental filtering interact in metacommunities. A metacommunity is “a set of local communities that are linked by dispersal of potentially interacting species” (Leibold *et al.* 2004). Not all patchy habitats house metacommunities: if all patches are completely isolated from one another, then species in different patches never have the opportunity to interact, and if all patches are completely mixed, then the patches together are a single community.

Ecologists have organized ecological processes into a metacommunity framework, a list of possible metacommunity types in which different ecological processes shape the species compositions of component communities (Leibold *et al.* 2004, Logue *et al.* 2011). The four metacommunity types are species-sorting, patch dynamics, mass effects, and neutral metacommunities. Species-sorting metacommunities assemble according to the niche concept of ecology: each component community has different environmental conditions and dispersal is not limiting, so each species persists in communities with suitable environmental conditions. In patch dynamics metacommunities, component communities have identical environmental conditions. Species in patch dynamics metacommunities have different dispersal and competitive abilities, and there is a tradeoff between dispersal and competition; good dispersers colonize patches first, and are then outcompeted by good competitors. Mass effects metacommunities resemble species-sorting metacommunities, but species have high enough dispersal rates that they can exist in patches with unsuitable environmental conditions. Finally, neutral metacommunities have identical habitat patches and identical

species dispersal and competitive abilities; diversity is maintained through stochastic births and deaths, and stochastic dispersal events among communities.

Ecologists often assign metacommunities to one of the four metacommunity types by correlating species compositions, environmental conditions, and geographic distance (space). Cottenie (2005) extended a variance partitioning technique of Legendre & Legendre (1998) to assign metacommunities on a space-environment axis: when community composition correlated with space independent of environmental conditions, Cottenie assigned a metacommunity to neutral or patch dynamics; when community composition correlated with environmental conditions independent of space, he assigned a metacommunity to species-sorting; and when community composition correlated with both space and environmental conditions, he assigned a metacommunity to mass effects. Microbiologists have taken advantage of this technique to assign microbial metacommunities to the ecological processes that shape metacommunity types. For example, Van der Gucht *et al.* (2007) examined bacterial community composition in ponds and lakes on a 2500 km spatial scale, and found significant correlations between environmental conditions and community compositions, but little evidence of dispersal limitation. They concluded that bacteria living in European lakes form a species-sorting metacommunity. There are two disadvantages to using variance partitioning to assign metacommunity type. The first is that it is a correlative technique, and is limited to presenting hypotheses in need of testing. The second is that variance partitioning studies tend to result in over 50% unexplained variation (*e.g.*, Van der Gucht 2007). Unexplained variation may be due to unmeasured environmental conditions, intertrophic interactions, or dispersal mechanisms that do not directly correlate with environmental distance (for

example, a vector may move propagules in a non-random pattern that researchers are unaware of).

### **Dispersal limitation and microbes**

Many researchers have hypothesized that dispersal operates fundamentally differently for macroorganisms and microorganisms. Historically, microbial ecologists assumed that all microbial species experience saturating dispersal, and that local environmental conditions filter local species from a global species pool (Baas Becking 1934, Finlay 2002). The only processes that this hypothesis (“the Baas Becking hypothesis”) invokes are deterministic environmental filtering and competition. The Baas Becking hypothesis is based on observations of similar microbial morphologies in distant habitats; researchers concluded from these observations that all microbial species have large enough populations to produce nearly infinite numbers of propagules, and therefore experience saturating dispersal. Recently, the “everything is everywhere; the environment selects” hypothesis has been discredited globally: there are population patterns in nature that can only be explained by invoking dispersal limitation (*e.g.*, Whitaker *et al.* 2003).

While microbial populations and species do not experience immediate saturating dispersal globally, they may experience saturating dispersal over smaller spatial scales or larger temporal scales. For example, all species in a microbial community covering a few kilometers, a few meters, or a few millimeters may produce enough propagules to reach the entire community. These species may then be subject to environmental filtering and competition. All species in a microbial community may also reach all locations given

enough time, although order of dispersal might lead to competitive advantages in early arriving organisms (priority effects, *e.g.*, Peay *et al.* 2012).

### **Fungi: macrobes and microbes**

Mycologists can compare small and large fungi with similar dispersal strategies and metabolisms to understand how ecological processes differ between macroorganisms and microorganisms. Individual fungal vegetative bodies have diverse sizes: they range from yeasts (3-8 x 5-10  $\mu\text{m}$ , reviewed in Kurtzman & Fell 1998) to large *Armillaria* underground mycelia (15 ha, Smith *et al.* 1992). For comparison, fungal bodies range in volume from bacteria sized to blue whale sized. Body size can also change throughout the life cycle of a fungal individual. Almost all fungi reproduce using microscopic spores, and large-bodied fungi only attain large sizes after having grown from one or two spores. Finally, all fungi—even those with very large vegetative bodies—interact with their environments on microscopic hyphal scales.

Fungal dispersal from one substrate to another can be passive, aided by vectors, or active. Most fungal spores are nonmotile and require transport by wind or water (*e.g.*, mushrooms frequently produce wind-dispersed basidiospores, Galante *et al.* 2011). Some fungi take advantage of vectors, *i.e.*, animals that move propagules of an organism between locations. Vectors can decrease an organism's dispersal limitation when they carry spores farther than wind or water can. Examples of insects and other animals that act as vectors for nonmotile fungi include aphids and beetles that move spores of rust fungi (Kluth *et al.* 2002), and flying squirrels that move spores of false truffles (Gabel *et*

*al.* 2010). A few fungal groups produce active zoospores, which can move distances of centimeters using flagella (*e.g.*, Chytrid pathogens of frogs, Piotrowski *et al.* 2004).

All fungi are heterotrophs, but they get energy from a variety of substrates. For example, fungi decompose most wood in temperate and tropical forests (Rayner & Boddy 1998); they also degrade many ephemeral substrates, like fruits and dung (*e.g.*, *Saccharomyces* on grapes, Fleet *et al.* 1984, *Pilobolus* on cow dung, Page 1962). In addition, many fungi gain energy through symbioses with a variety of plants and animals. They can be pathogens (*e.g.*, Laboulbeniales on insects, Weir & Hammond 1997), commensals (*e.g.*, plant endophytes, Arnold 2007), and mutualists (*e.g.*, mycorrhizal fungi, Landeweert *et al.* 2001, lichens, Brodo *et al.* 2001).

### **Pitcher plants: model metacommunities**

I investigated fungi that inhabit *Sarracenia* carnivorous plant pitchers, and focused on two *Sarracenia* species: *S. purpurea* and *S. rosea*. *Sarracenia* species produce pitcher-shaped leaves (pitchers). When young, *S. purpurea* and *S. rosea* pitchers are sealed closed and sterile (Peterson *et al.* 2008). As pitchers mature, they open and collect rainwater; both microorganisms and macroorganisms live in opened pitchers. Pitchers also attract insects with extrafloral nectaries, and some insects fall in pitchers and drown (Bennett & Ellison 2009). Invertebrates, protists, and other organisms that inhabit pitchers shred and decompose insect prey, and these pitcher-inhabiting organisms form a food web with bacteria at the bottom and insect larvae at the top (reviewed in Ellison *et al.* 2003). Fungi, especially yeasts, also inhabit pitchers, and may occupy similar niches to bacteria. Ecologists have recently shown an interest in the biogeography of the bacteria

that inhabit pitcher plants. For example, Peterson *et al.* (2008) showed that pitcher-inhabiting bacteria have community structure over tens of kilometers; Koopman *et al.* (2010) showed that bacterial communities change in pitchers over the growing season; Kreiger & Kourtev (2011) showed that subhabitats within pitchers house distinct bacterial communities; and Koopman & Carstens (2011) showed that bacterial community structure reflects host phylogeographic structure over tens to hundreds of kilometers.

*Sarracenia purpurea* and *S. rosea* occupy bogs or wet savannas, and together range from the United States Gulf Coast to southern Canada. *Sarracenia purpurea* ranges from Georgia north to Newfoundland, and west through the northeastern United States and southern Canada to northeastern British Columbia. *Sarracenia rosea* has a more limited distribution, along the Gulf Coast from Louisiana to the Florida panhandle (Naczi *et al.* 1999). Taxonomists have only recently split *S. rosea* from *S. purpurea*; *Sarracenia rosea* is often included in investigations of *S. purpurea* and its associated organisms over broad spatial scales (*e.g.*, Buckley *et al.* 2010).

Organisms living in *Sarracenia* pitchers live in a hierarchically structured patchy habitat. Organisms within a pitcher can interact with each other through competition, for example, but only interact with organisms in another pitcher after dispersing to that pitcher. Organisms inhabiting separate islands on a bog are also separated from other islands by space and must first disperse in order to interact. Finally, both *Sarracenia* species occupy patchy habitats on the landscape, and organisms in individual *Sarracenia* populations must disperse to encounter each other. Organisms may form a metacommunity at each level of spatial organization.



## Questions and hypotheses

In this thesis, I ask how dispersal influences fungal diversity in pitcher plants, and how dispersal interacts with other ecological processes. Over the course of my dissertation work, I looked for patterns consistent with the presence of dispersal limitation or dispersal saturation on local and continental spatial scales. I also tested for dispersal limitation and dispersal saturation local spatial scales. Finally, I looked for interactions between dispersal, competition, and environmental filtering on single pitcher and continental scales.

To understand if fungi experience dispersal limitation at local scales, I observed whether pitcher fungi appear in pitchers immediately after pitchers open. I isolated three target yeast species from pitchers, and subsequently observed their arrivals in pitchers over a single bog habitat. If all target species do not arrive in pitchers as soon as pitchers open, then fungi do not experience saturating dispersal on local spatial scales. I also looked for variation in arrival times among yeast species. I observed the day that each of the three target yeasts appeared in pitchers, and interpreted different appearances either as different dispersal times or as environmental filtering; a yeast that does not appear early in a pitcher's life span may not be able to survive in the niches provided by young pitchers. To distinguish between the variable dispersal limitation and environmental filtering hypotheses, I tested for environmental filtering by inoculating young pitchers with a yeast that appears in pitchers late in the growing season. Survival of a late-arriving yeast in young pitchers indicates that pitcher environments do not prevent establishment of late-appearing yeasts when pitchers are young.

I also looked for correlative evidence of dispersal limitation and dispersal saturation on continental spatial scales. I estimated population structure of the most abundant pitcher plant yeast, *Candida glabrata*, and interpreted that there is saturating dispersal among individuals from locations with interbreeding genotypes. I also looked for fungal community structure over space, and interpreted that different locations containing the same community had saturating dispersal. However, locations with different communities may experience dispersal saturation, but be structured by processes other than dispersal (*e.g.*, environmental filtering).

Finally, I looked for interactions between dispersal and other processes on pitcher and continental scales. I asked if high dispersal could give a competitive advantage to yeasts in pitcher-like microcosms. I also looked at correlations between community composition and environmental conditions on a continental spatial scale. If environmental filtering and competition shape fungal communities, I expected to observe a correlation between environmental conditions and fungal community structure when I controlled for geographic distance among communities.

## **CHAPTER 2**

**Fungal species composition changes over space and time**

## Abstract

Fungi are often the primary decomposers and nutrient cyclers in an ecosystem, and they can also facilitate or limit other organisms as mutualists and pathogens. However, ecologists do not yet fully understand how fungal species and populations are distributed over space and time, or how ecological processes shape fungal diversity. To better understand fungal diversity, we surveyed fungal taxa in pitchers of *Sarracenia purpurea* and *S. rosea* carnivorous plants. We used 454 sequencing to enumerate taxon diversity in five locations on a 4000 km spatial scale and over two months at one location. Fungal taxon numbers in pitchers peak early in the *Sarracenia* growing season and decline as the season progresses, and taxon composition is significantly different at different times in the season. There were also four distinct fungal communities in the five sampled locations; thousands, but not hundreds, of kilometers separated distinct communities. We propose processes causing observed diversity patterns, including environmental restrictions, dispersal, and biotic interactions, and identify correlations to support each process.

## Introduction

The central goals of ecology include both understanding the numbers and identities of species in nature, and understanding the processes that maintain them. Ecologists understand biodiversity patterns of large charismatic organisms like vertebrates better than they understand biodiversity patterns of microorganisms, including fungi (Blackwell 2011). Only about 97 000 of the estimated 1.5 million or more fungal species on earth have been described (Kirk *et al.* 2008, Hawksworth 1991).

Fungal diversity over space and time shapes community ecological processes and ecosystem function. For example, local pathogen diversity can limit the ranges of trees in tropical forests (Gilbert & Webb 2007, Liu *et al.* 2011). Identities of wood decomposing fungi on a single substrate change over time and control rates of decomposition (Holmer & Stenlid 1997, Boddy *et al.* 1989). Arbuscular mycorrhizal fungal (AMF) communities change over both space and time, and the different identities of AMF symbionts cause changes in host biomass (Yang *et al.* 2012, Pringle & Bever 2002, Bever *et al.* 1997). These examples illustrate that patterns of fungal diversity can have widespread impacts; we describe fungal diversity in a model system and provide a first step to understanding how fungal community processes and functions change over space and time.

We describe fungal species diversity in carnivorous plant pitchers of the genus *Sarracenia*. We targeted two *Sarracenia* species with different ranges across the United States and Canada: *S. purpurea* and *S. rosea*. *Sarracenia purpurea* ranges from Georgia north to Newfoundland, and west through the northeastern United States and southern Canada to northeastern British Columbia. *Sarracenia rosea* has a more limited distribution, along the Gulf Coast from Louisiana to the Florida panhandle (Naczi *et al.*

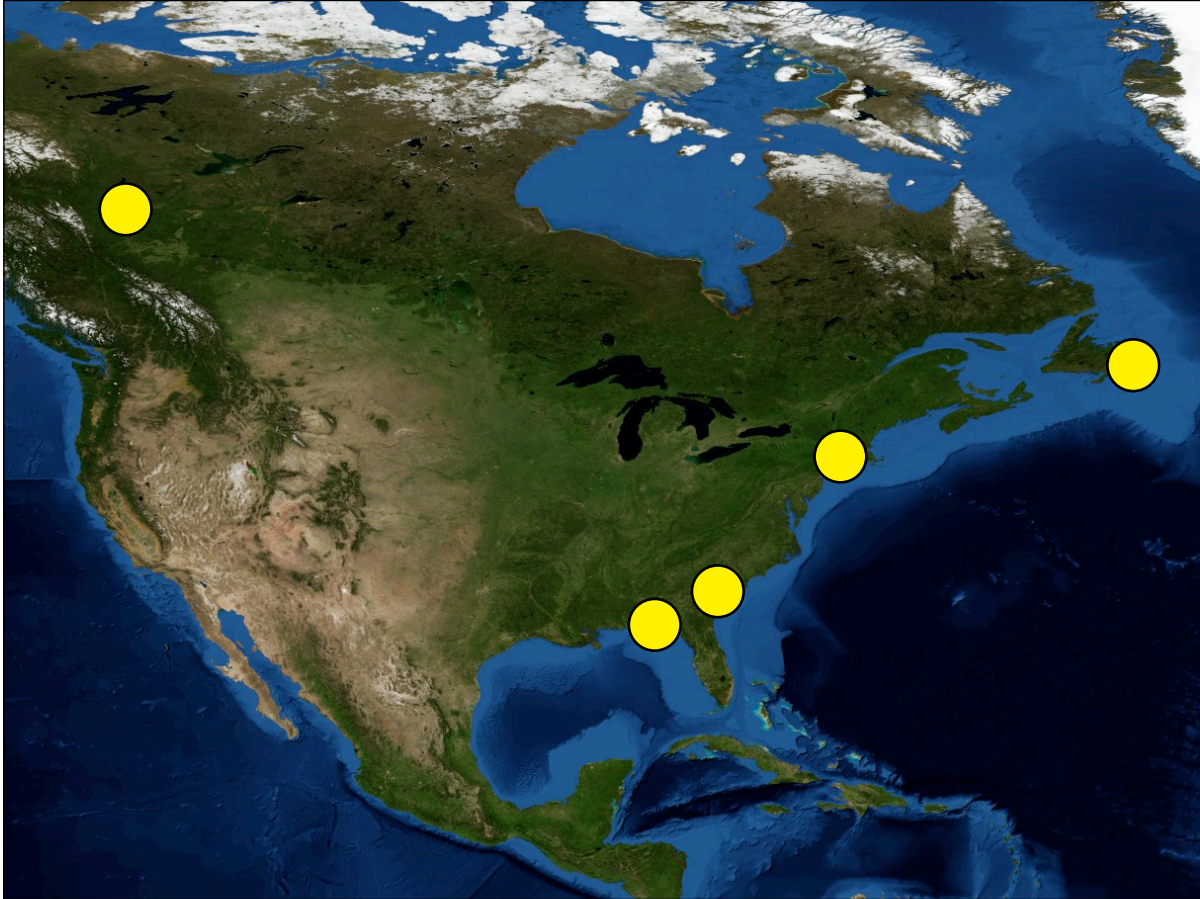
1999). Taxonomists have only recently split *S. rosea* from *S. purpurea*; *Sarracenia rosea* is often included in investigations of *S. purpurea* and its associated organisms over broad spatial scales (e.g., Buckley *et al.* 2010). Both plants have similar morphologies, and develop pitcher-shaped modified leaves (pitchers). Pitchers fill with rainwater and attract insects, which occasionally fall into pitchers and drown. In both species, pitchers develop aquatic food webs comprising arthropods, protists, and other small animals and microorganisms (reviewed in Ellison *et al.* 2003). These food webs shred and decompose insect prey. Fungi are easily cultured from pitchers, but the ecological functions of fungi in pitcher ecosystems are unknown; they likely perform many functions, including decomposition and plant and animal symbiosis. For this study, we enumerated fungal diversity in pitchers using high-throughput 454 sequencing. We described fungal species composition on a continental spatial scale, and, at one location, over a *S. purpurea* growing season.

We asked two questions about fungal community diversity in pitcher plants: 1) How do *Sarracenia* fungal communities differ across the range of their host plant? 2) How do *Sarracenia* fungal communities change over a single growing season? We follow the answers to these questions with speculation on the processes that might drive fungal diversity patterns and the influence of fungal diversity on pitcher ecosystems.

## **Materials and methods**

### *Field collections: geographic sampling*

In July and August of 2010, we sampled from *Sarracenia* populations once at each of five locations in the United States and Canada (Figure 2.1, Table 2.1). We



*Figure 2.1: Map of the United States and Canada showing the five sampled locations.  
Image courtesy of NASA.*

Table 2.1: Details on sites and fungal community sampling conducted in the United States and Canada in the summer of 2010.

Site	Location	Sampling date	Temperature (°C)	Precipitation (mm)	Plants sampled
British Columbia	Parker Lake Ecological Reserve	July 19, 2010	-0.7	451.7	5
Newfoundland	La Manche Provincial Park	August 19, 2010	4.7	1513.7	11
Massachusetts	Harvard Forest	July 13, 2010	8.8	1202.8	7
Georgia	Tattnall County	July 5, 2010	19.3	1252.8	6
Florida	Apalachicola National Forest	July 7, 2010	19.4	1604.0	5

sampled from a single *S. purpurea* population in each of British Columbia, Newfoundland, Massachusetts, and Georgia, and from two *S. rosea* populations 10 km apart in Florida. Habitat types included *Sphagnum* bogs (British Columbia, Newfoundland, and Massachusetts) and pine savanna (Georgia and Florida). Distances between populations ranged from 10 to 4630 km. We used average annual temperature and precipitation data (1971-2000) at each site to test for relationships between coarse environmental conditions and fungal community composition (NOAA 2012, Environment Canada 2002). We chose temperature and precipitation because they represented coarse continent-scale variation in climate. Temperature and precipitation were only available from Environment Canada as averages from 1971-2000; for consistency, we used averages from the same year range for locations in the United States.

To control for the effect of pitcher age on fungal communities, we collected water from differently aged pitchers at each plant. *Sarracenia* pitchers develop one by one, as a rosette, about 20 days apart throughout the growing season (Fish & Hall 1978); pitchers



from different parts of a rosette have different ages. Two pitchers can be as close in age as 20 days. Pitchers occasionally persist from one growing season to the next, and two pitchers can be as distant in age as over a year (Miller & Kneitel 2005). We selected plants with at least four pitchers and sampled from all pitchers on a plant. If the plant consisted of more than ten pitchers, we drew an imaginary line through the center of the plant, and sampled all pitchers along the line; in this way we sampled young pitchers from the center of the plant's rosette, old pitchers from the rosette's periphery, and intermediate aged pitchers between the rosette's center and periphery. The number of pitchers sampled per plant ranged from 4 to 13, and we combined all pitcher samples for each plant before sequencing. We removed water from each pitcher using a sterile plastic transfer pipette. First, we mixed the water inside a pitcher by pipetting up and down. We then removed about 0.25 ml of pitcher water, excluding large insect parts, and mixed the pitcher water with 0.25 ml of 2x CTAB buffer (100 mM Tris pH 8.0, 1.4 M sodium chloride, 20 mM Ethylenediaminetetraacetic acid disodium salt dihydrate, 2% cetyl trimethylammonium bromide). Samples were flash-frozen within six days of collection and stored at -20 or -80°C until DNA extraction.

*Field collections: temporal sampling*

Starting in May of 2009, we sampled pitcher water over a single growing season from pitchers in the Massachusetts location. We identified 43 unopened pitchers, recorded the date each pitcher opened, and sampled water from each pitcher 6-9 days, (hereafter referred to as one week), 34-42 days (one month), and 66-74 days (two months; only 33 of the original 43 pitchers were intact at this timepoint) after it opened.

We removed water from each pitcher at each timepoint as described above, except that samples were frozen within five hours of collection. We screened each pitcher sample for the presence of fungal sequences using a PCR assay.

Prior to the PCR assay, we extracted DNA from each sample. We thawed and centrifuged frozen samples at 16.1 g for 10 min, and removed the supernatant from each pellet. Then we suspended each pellet in 200  $\mu$ L of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM sodium chloride, 10 mM Tris, and 1 mM Ethylenediaminetetraacetic acid; Hoffman 1997). We mixed each suspension with about 200  $\mu$ L of 0.5 mm glass beads and 200  $\mu$ L 25:24:1 chloroform:phenol:isoamyl alcohol. We vortexed each mixture for 2 min, and then centrifuged it for 5 min at 16.1 g. After centrifugation, we removed the aqueous layer and mixed it with 2.5 volumes of 95% ethanol and 0.1 volume of 3M sodium acetate (Sambrook & Russell 2001); we incubated each aqueous layer mixture at -20°C for at least three hours. Next, we centrifuged each aqueous layer mixture for 15 min at 16.1 g, and removed the supernatant. Finally, we washed each pellet with 0.5 ml 70% ethanol, centrifuged each mixture for 10 min at 16.1 g, removed the supernatant, and resuspended each pellet in 50  $\mu$ L water.

Next, we attempted to amplify fungal DNA from extracts using the fungal-specific primer pair ITS1F and ITS4 (Gardes & Bruns 1993, White *et al.* 1990). Each PCR reaction was composed of 7.9  $\mu$ L water, 0.1  $\mu$ L GoTaq® Flexi polymerase (Promega), 5  $\mu$ L Flexi buffer with green dye added, 5  $\mu$ L 5x CES (combinatorial PCR enhancer solution: 2.7 M betaine, 6.7 mM dithiothreitol, 6.7% dimethyl sulfoxide, 55  $\mu$ g/mL bovine serum albumin; Ralser *et al.* 2006), 5  $\mu$ L nucleotide mix, 2  $\mu$ L magnesium chloride, 1  $\mu$ L of 10  $\mu$ M of each primer, and 1  $\mu$ L template DNA extract. All reactions

were cycled on a Biorad iCycler or myCycler using denaturing, annealing, and extension temperatures of 95, 55, and 72 °C, respectively. We denatured for 85 s, then ran 13 cycles of 35 s denaturing, 55 s annealing, and 45 s extension, followed by 13 cycles that were identical but had a 2 min extension, and finally 9 cycles with a 3 min extension. We ran a subsequent 10 min extension. Two  $\mu$ L of each PCR product were visualized on 1% agarose gels stained with SYBR® Safe dye (Invitrogen) and photographed using a U:genius gel documenting system (Syngene) and a Stratagene transilluminator.

Photographs of gels were scored for presence or absence of a band. Bands that were too faint to reliably score were run a second time with 6  $\mu$ L of PCR product per well.

Presence of a band on a gel indicated the presence of detectable fungal DNA in a water sample. Of the 43 pitchers (33 at two months), fungal DNA was present in 91% of pitchers after one week, 95% after one month, and 73% after two months. We chose 17 of the original 43 pitchers with detectable fungal DNA at all three timepoints for 454 sequencing. We sequenced samples from these pitchers at all timepoints. In total, we sequenced and analyzed DNA from 51 pitcher-timepoint combinations.

#### *454 sequencing*

We prepared two amplicon pools for 454 sequencing: one pool contained only geographic samples, and the other contained only temporal samples. Each amplicon pool consisted of amplicons of the ITS2, 5.8s, and ITS1 ribosomal regions; we targeted regions amplified by the fungal specific ITS1F/ITS4 primer pair. Individual samples in each amplicon pool were tagged using 10-bp multiplex identifier (MID) tags (454 Life Sciences Corporation 2009) to distinguish samples. For temporal sampling, each sample

is an individual pitcher-timepoint combination. For geographic sampling, each sample is all pitchers from an individual plant combined. In total, we sequenced DNA from 239 pitchers combined into 38 plant samples for the geographic data set, and 51 pitcher-timepoint combinations for the temporal data set.

We extracted fungal DNA and amplified it using the extraction protocol and PCR recipe described above, except that Gotaq® Hotstart polymerase (Promega) was used instead of Flexi polymerase, and we used 50 µM instead of 10 µM of the reverse primer. We used a forward primer consisting of (in order from 5' to 3') the 454 "A" primer (CCATCTCATCCCTGCGTGTCTCCGACTCAG) concatenated with a 10-bp multiplex tag, and ITS4; we used a reverse primer consisting of the 454 "B" primer (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG) concatenated with ITS1F. Multiplex tags were unique to each sample. Reactions were cycled at 95 °C for 15 min; 30 cycles of 95 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min; and a final extension of 72 °C for 8 min. PCR products were purified using Agencourt® *AMPure*® XP (Beckman Coulter) and quantitated using a Qubit® dsDNA HS Assay (Invitrogen) according to the manufacturers' instructions.

To make geographic samples representing all sampled pitchers for each plant, we combined equimolar concentrations of PCR products from each pitcher in a plant together. To make temporal samples representing several PCR replicates for each pitcher-timepoint combination, we combined equimolar concentrations of the products of each of three PCR reactions from each pitcher-timepoint DNA extract. For each sequencing pool (geographic and temporal), we pooled equimolar concentrations of PCR products from either each plant sample (many pitchers combined) or each pitcher-timepoint

combination. The Duke Genome Sequencing & Analysis Core Resource carried out 454 sequencing: geographic and temporal pools were each sequenced on separate one-eighths of a 454 Titanium sequencing run.

#### *454 sequence processing*

We processed 454 sequencing runs using QIIME 1.3.0 (Caporaso *et al.* 2010). Low quality sequences were removed and remaining sequences were assigned to their MID barcodes using the default QIIME quality filtering settings. Primers and barcodes were trimmed from each sequence and sequences shorter than 200 and longer than 1000 bp were removed from each data set. Sequences were denoised using the QIIME denoiser.

We reduced chimeric sequences by trimming the 5.8s and ITS1 portions from all sequences and only analyzing the ITS2 portion. The 5.8s ribosomal region lies between the ITS1 and ITS2 spacers, and is conserved among fungi relative to the spacers. Because the 5.8s region is conserved, we expected most chimeric sequences to form in the 5.8s region and to be composed of ITS1 and ITS2 sequences from different templates (Nilsson *et al.* 2010a). While chimera detection software for fungal ITS sequences exists (Nilsson *et al.* 2010a), it requires longer sequences than the ones we produced. We extracted ITS2 subunits using the Fungal ITS Extractor (Nilsson *et al.* 2010b).

We chose operational taxonomic units (OTUs) using the uclust method in QIIME, which forms OTU clusters around seed sequences taken from the input dataset, at 97% similarity. All singleton OTUs were discarded. The longest sequence in each remaining cluster was retained as a representative sequence.

Of the total of 152 667 sequences produced for the geographic data set, 23 663 were discarded for having lengths less than 200 or more than 1000 bp, and 48 024 were discarded because either they had low quality, they did not have matching barcodes, the ITS2 subunit could not be extracted, or they were singleton OTUs. In total, we retained 80 980 sequences from the geographic data set for further analysis, and the number of sequences per plant sample ranged from 292 to 4490 in the 38 samples.

Of the total of 141 424 sequences produced for the temporal data set, 27 632 were discarded for having lengths less than 200 or more than 1000 bp and 14 160 were discarded because either they had low quality, they did not have a matching barcode, the ITS2 subunit could not be extracted, or they were singleton OTUs. In total, we retained 99 632 sequences from the temporal data set for further analysis, and the number of sequences per pitcher-timepoint combination ranged from 253 to 4365 in the 51 samples.

To assign taxonomy, we performed a MEGAN analysis on the top ten hits from the NCBI BLAST nucleotide database extracted using BLAST 2.2.25+ (Huson *et al.* 2011, Zhang *et al.* 2000), and the default MEGAN settings. MEGAN takes files of sequences and BLAST outputs, and assigns each sequence to a taxon based on the lowest taxonomic level shared by all BLAST hits above a threshold bit score for that sequence. We discarded OTUs matching organisms not in the kingdom Fungi (plant, animal, and protist sequences), and we assumed that OTUs with no BLAST matches or matching unassigned fungal environmental sequences were fungal sequences not yet identified in the NCBI database because we used fungal specific primers. We retained unassigned OTUs for diversity measurements, but did not include them in taxonomy summary plots. We reviewed the taxon assignments output by MEGAN manually, and we filled in

higher-level classifications (*e.g.*, order or class) using Index Fungorum (<http://www.indexfungorum.org/>) when an OTU was assigned to a genus but not higher-level classifications. We attempted to assign all taxa to genera; if it was not possible to assign a taxon to a genus, we assigned it to the lowest order taxonomic group possible.

### *Statistical analyses*

We compared numbers of OTUs observed and OTU composition among locations and timepoints using rarefied data sets. We chose rarefied numbers of OTUs observed instead of other diversity measures (*e.g.*, Chao1, Shannon Index) because these other diversity measures extrapolate total diversity. Extrapolation of 454 data sets can be unreliable because extrapolation can amplify sequencing errors (Gihring *et al.* 2012). We first discarded the 4 samples from the geographic data set and 8 samples from the temporal data set containing less than 1000 sequences each, and then rarefied the remaining samples to the lowest OTU count in each data set. We rarefied to 1109 sequences at each location or 1140 sequences at each pitcher-timepoint combination.

We compared alpha diversity (number of taxa observed in a location) among locations using one-way analysis of variance (ANOVA), and among timepoints using repeated measures ANOVA controlling for pitcher. ANOVAs were performed using R 2.14.1 (R Development Core Team 2011). Although we sampled from 17 pitchers for temporal patterns, we discarded samples with fewer than 1000 sequences; to maintain a balanced design, we only included data from 10 pitchers in the repeated measures ANOVA. We produced species-accumulation curves with 95% confidence intervals for

each full data set using EstimateS 8.2.0 (Colwell 2005), and the Mao Tau estimator of observed species (Colwell *et al.* 2004).

We visualized species compositions in each sample using nonmetric multidimensional scaling (NMDS) and tested for differences in species composition among locations or timepoints using analysis of similarity (ANOSIM), using the rationale of Sthultz *et al.* (2009). Briefly, NMDS is an ordination method that ranks similarities of pairs of samples, then randomly places samples on a two-dimensional plot and iteratively moves more similar communities closer together (Clarke 1993). We only used NMDS to visually summarize our data; NMDS plots were created using the vegan 2.0-3 package in R (Oksanen *et al.* 2008). ANOSIM is a nonparametric statistical test for spatial or temporal structure: first, the algorithm calculates a statistic,  $R$ , between -1 and 1, where  $R = 1$  if all pairs of samples within a group are more similar than pairs of samples from different groups; next, the algorithm repeatedly shuffles the labels on samples and calculates  $R$  for shuffled data sets; finally, the algorithm calculates a p-value based on the percentage of shuffled data sets with larger  $R$  than the original data set (Clarke 1993). Pairwise ANOSIM corrects p-values for multiple comparisons. ANOSIMs and pairwise ANOSIMs were conducted with Primer 5 (Clarke & Gorley 2006). We transformed each rarefied OTU sequence abundance matrix to reflect taxon presences and absences instead of sequence abundance; biases introduced through PCR, sequencing, and the number of ribosomal copies in a cell's genome can influence sequence abundance (Amend *et al.* 2010a). We therefore consider 454 presence-absence data to be more reliable than sequence abundance data. However, analyses conducted using OTU abundance matrices are provided in Appendix 1; sequence abundances were



standardized within samples (plants or pitcher-timepoint combinations) so that each sample had the same total relative abundance, but abundances of each OTU varied within a sample. Relativized abundances were then square-root transformed. We used the Bray-Curtis distance metric for all ANOSIM and NMDS analyses.

We detected many taxa from the Agaricomycetes in our data sets, and produced additional NMDS plots and ANOSIMs with subsets of each data set that did not include Agaricomycete or unassigned taxa. The Agaricomycetes are a class of Basidiomycetes (including mushrooms) that produce large fruiting bodies and numerous spores (Hibbett *et al.* 2007). Observed Agaricomycete sequences are either from Agaricomycete fungi that grow in pitchers or are from spores temporarily found in pitchers. To be conservative, we removed unassigned OTUs in addition to Agaricomycete OTUs from this analysis. NMDS plots and ANOSIMs from subsets were conducted as described above. After removing OTUs, we discarded the 7 plant samples from the geographic data set and 21 pitcher-timepoint samples from the temporal data set with fewer than 1000 sequences. We then rarefied the geographic and taxonomic data sets to 1033 and 1276 sequences respectively.

To understand the relationships between species composition, spatial, and environmental factors, we correlated OTU dissimilarity with geographic and environmental distance using Mantel and partial Mantel tests. Mantel tests are analogous to linear regression: they correlate community distance between pairs of samples with geographic or environmental distance while correcting for spatial autocorrelation (Mantel 1967, Fortin & Gurevitch 2001). Mantel tests first calculate Mantel's R, a statistic analogous to Pearson's  $r$ , based on the sum of products of corresponding cells in each of

two distance matrices (community composition distance, geographic distance, or environmental distance). Mantel's  $R$  ranges from -1 to 1, and  $R = 1$  when all pairs of geographically close (or environmentally similar) samples are more similar to each other than pairs of geographically distant (or environmentally different) samples are. Significance is computed by repeatedly shuffling all cells in one matrix, calculating Mantel's  $R$  on shuffled matrices, and calculating a p-value based on the percentage of shuffled data sets with Mantel's  $R$  greater than that calculated for the unshuffled data set. Partial Mantel tests compare two matrices while controlling for a third: they are Mantel tests calculated on the residuals of community distance and environmental difference (in the case of our data) on geographic distance. In this way, partial Mantel tests indicate correlation between community and environmental distances when geographic distance is controlled. We computed OTU dissimilarity on each rarefied presence-absence matrix using Jaccard dissimilarity in the *vegan* library in R, and calculated geographic distance between sampling points using the *fields* library in R (Furrer *et al.* 2012). All Mantel tests were conducted in the *vegan* library in R with 999 permutations.

## Results

### *454 sequence summary*

In total, geographic sequences produced 696 OTUs, of which 497 were assigned to fungal taxa; 3 were discarded because they matched non-fungal sequences; and 196 were not assigned. Of the 497 fungal taxa in the geographic data set, 35% were Basidiomycota, 59% Ascomycota, and 6% basal fungal lineages. Temporal sequences produced 553 OTUs, of which 379 were assigned to fungal taxa; fifteen were discarded

because they matched non-fungal sequences; and 159 were not assigned. Of the 379 fungal taxa in the temporal data set, 52% were Basidiomycota, 43% Ascomycota, and 5% basal fungal lineages.

### *Fungal species alpha diversity*

We observed a decline in alpha diversity (defined as OTU numbers observed at a timepoint or location) with pitcher age (Tables 2.2 and 2.3), and no change in alpha diversity among locations (Tables 2.4 and 2.5). The average number of OTUs observed at each timepoint declined significantly over time ( $p = 0.03$ ). The average number of OTUs observed at each location ranged from 41.4 in Florida to 51.8 in Newfoundland, and there were no detectable differences among sites ( $p = 0.84$ ). Our sampling did not saturate species accumulation curves for either the geographic or temporal data sets (Figure A1.1).

*Table 2.2: Numbers of taxa observed at each timepoint.*

Timepoint	Average rarefied OTUs observed	standard deviation
week	43.5	14.8
month	28.6	14.6
two months	23.1	11.3

*Table 2.3: ANOVA table for alpha diversity among timepoints.*

Source	Degrees of freedom	Sum of squares	Mean squares	F value	p
timepoint	2	2240	1120.0	4.488	0.03
Residuals	18	4493	249.6		

*Table 2.4: Numbers of taxa observed at each site.*

Site	Average rarefied OTUs observed	standard deviation
British Columbia	46.0	16.5
Newfoundland	51.8	17.9
Massachusetts	50.1	21.0
Georgia	50.3	13.1
Florida	41.4	16.7

*Table 2.5: ANOVA table for alpha diversity among geographic locations.*

Source	Degrees of freedom	Sum of squares	Mean squares	F value	p
Geographic location	4	440	110.0	0.358	0.84
Residuals	29	8909	307.2		

#### *Community composition over space*

Taxon compositions differed significantly across locations (ANOSIM  $R = 0.85$ ,  $p < 0.001$ , Figure 2.2a). Each site contained a distinct fungal assemblage, except Florida and Georgia; assemblages in these two locations were not significantly different from one another (pairwise ANOSIM  $R = 0.22$   $p = 0.056$ ; all other pairwise  $R > 0.86$ ,  $p < 0.008$ ). Sequence abundance data follow the same general pattern (Figure A1.2). Subsets of the data without Agaricomycete or unassigned sequences also follow the same general pattern (Figure A1.3). We observed a decay in assemblage similarity with distance (Mante's  $R = 0.47$ ,  $p = 0.001$ ), although whether or not two samples came from the same location explained assemblage similarity better than geographic distance did (Mantel's  $R = 0.70$ ,  $p = 0.001$ ). When the effects of geographic distance were removed, assemblage similarity correlated with difference in average yearly temperature between sites (Mantel's  $R = 0.59$ ,  $p = 0.001$ ), but not average yearly precipitation (Mantel's  $R = -0.39$ ,  $p = 1$ ). Ascomycete taxa dominated at all sites, and were a larger component of southern sites than they were of northern sites (Figure 2.2b).

#### *Community composition over time*

Taxon compositions differed significantly among timepoints (ANOSIM  $R = 0.41$ ,  $p < 0.001$ ; Figure 2.3a). Week old fungal communities had significantly different compositions from older fungal communities (pairwise ANOSIM  $R = 0.58$ ,  $p < 0.001$

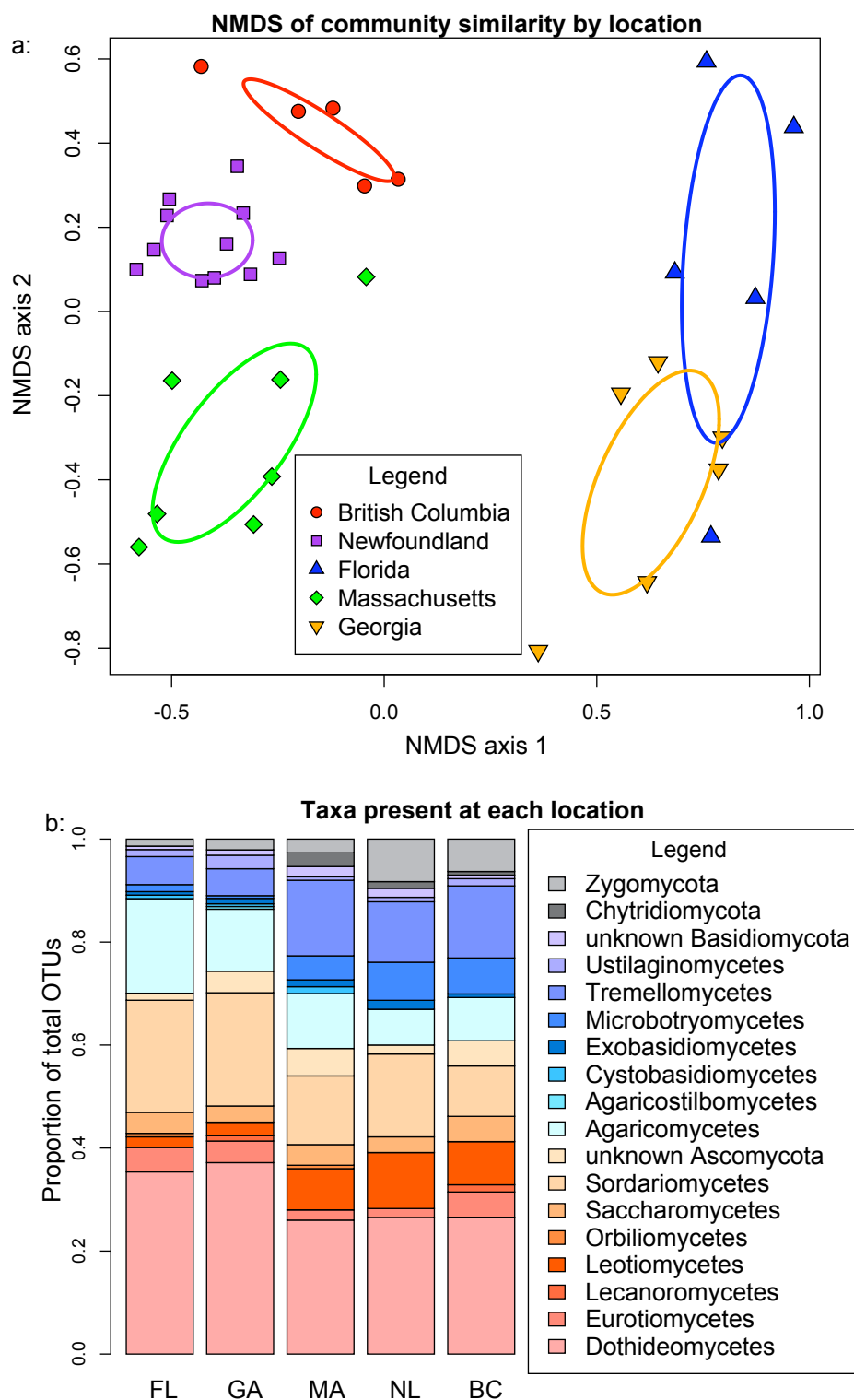


Figure 2.2: Fungal diversity at five locations. 2.2a: NMDS ordination of similarities in OTU composition between locations. Ellipses depict 95% confidence intervals of centroids of each group. 2.2b: Percentages of taxa in each fungal phylum or class at each location. Ascomycetes are in red and orange; Basidiomycetes are in blue; and basal fungal lineages are in gray.

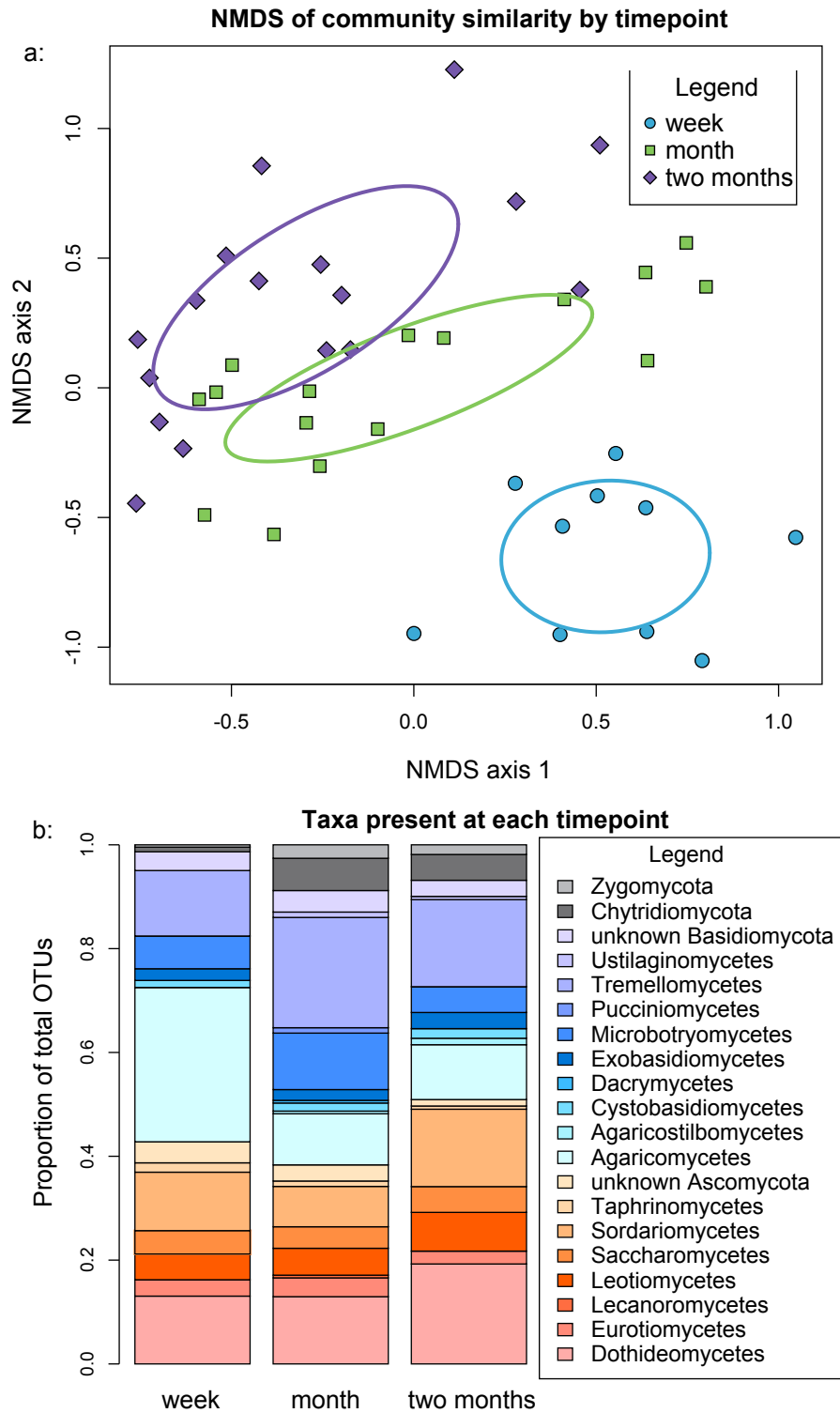


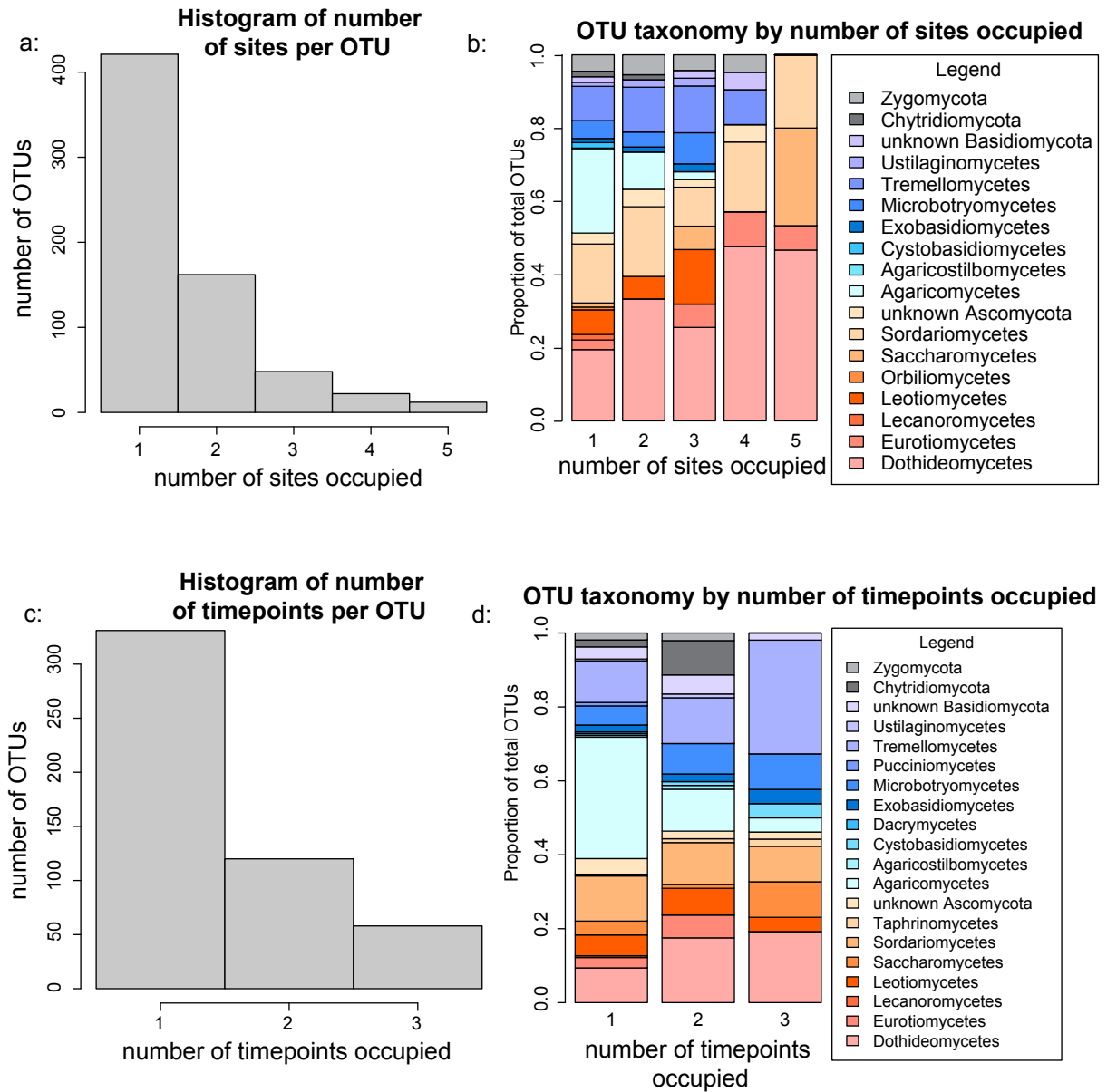
Figure 2.3: Fungal diversity at three timepoints. 2.3a: NMDS ordination of similarities in OTU composition between timepoints. Ellipses depict 95% confidence intervals of centroids of each group. 2.3b: Percentages of taxa in each fungal phylum or class in each timepoint. Ascomycetes are in red and orange; Basidiomycetes are in blue; and basal fungal lineages are in gray.

between week and month timepoints, ANOSIM  $R = 0.70$ ,  $p < 0.001$  between week and two-month timepoints); month and two-month communities were not significantly different from each other (pairwise ANOSIM  $R = 0.08$ ,  $p = 0.052$ ). Sequence abundance data produced a similar trend, although in these data, month and two-month communities were significantly different from each other (pairwise ANOSIM  $R = 0.08$ ,  $p = 0.037$ , Figure A1.4). Subsets without Agaricomycete or unassigned taxa showed the same trend when data were presence-absence transformed, except that the month and two-month timepoints were no longer significantly different from each other (pairwise ANOSIM  $R = 0.01$ ,  $p = 0.40$ , Figure A1.5a). Subsets without Agaricomycetes or unassigned taxa were not significantly different when we used sequence abundance data (ANOSIM  $R = 0.06$ ,  $p = 0.17$ , Figure A1.5b). We observed Basidiomycete taxa more frequently than taxa from other phyla at the week and month timepoints, and Ascomycete taxa more frequently at the two-month timepoint (Figure 2.3b).

#### *Widespread taxa over space and time*

Only Ascomycete taxa occupied all five locations, but both Basidiomycetes and Ascomycetes occupied all three timepoints (Figures 2.4b, 2.4d). Most taxa (63% of the geographic taxa and 65% of the temporal taxa, Figures 2.4a, 2.4c) occupied a single site or timepoint. Only 2% of geographic taxa occupied all five sites. Genera occupying all five sites included *Cercospora*, *Cladosporium*, *Epicoccum*, *Phoma*, *Candida*, *Bionectria*, *Fusarium*, and *Lecanicillium*.

In contrast, 11% of temporal taxa occupied all three timepoints; these taxa included both Basidiomycetes and Ascomycetes (Figure 2.4d). Genera occupying all



*Figure 2.4: Widespread and endemic OTUs by number of locations or timepoints occupied. 2.4a: Histogram of number of geographic locations occupied by OTUs. 2.4b: Taxonomic breakdown by number of locations occupied. Ascomycetes are in red and orange; Basidiomycetes are in blue; and basal fungal lineages are in gray. 2.4c: Histogram of number of timepoints occupied by OTUs. 2.4d: Taxonomic breakdown by number of timepoints occupied. Ascomycetes are in red and orange; Basidiomycetes are in blue; and basal fungal lineages are in gray.*



three timepoints included *Cladosporium*, *Pseudocercospora*, *Ramichloridium*, *Aureobasidium*, *Kabatiella*, *Rhizosphaera*, *Epicoccum*, *Venturia*, *Candida*, *Sirococcus*, *Acremonium*, *Bionecria*, *Fusarium*, *Hypocrea*, *Taphrina*, *Resnicium*, *Ganoderma*, *Rhodotorula*, *Exobasidium*, *Malassezia*, *Sporidiobolus*, *Cryptococcus*, *Bulleromyces*, *Tremella*, and *Kriegeria*.

## **Discussion:**

### *Fungal community patterns over space*

We observed four distinct fungal communities in the five sampled locations: assemblages grouped into southern (Florida and Georgia), Massachusetts, Newfoundland, and British Columbian communities. Fungal communities differentiated over thousands, but not hundreds, of kilometers (the Florida and Georgia sites are ~350 km from each other). Pitcher plant fungi have similar community structure on continental scales to endophytic and endolichenic fungi. For example, U'Ren *et al.* (2012) found that leaf endophytic and endolichenic fungal communities were distinct over thousands of kilometers, ranging from Alaska to Florida. In addition, Hoffman & Arnold (2008) found that endophyte communities are distinct between Arizona and North Carolina, and Davis & Shaw (2008) found that liverwort endophyte community similarity decays with distance on global scales in a study targeting North America, Europe, and Oceania.

Metacommunity theory predicts that competition, environmental filtering, and dispersal structure community composition in isolated patches like pitchers (isolated by centimeters to thousands of kilometers) and pitcher plant habitats (isolated by tens to thousands of kilometers); geographic distance, environmental variation, or both can

correlate with these ecological processes (Liebold *et al.* 2004, Cottenie 2005).

Geographic distance is a proxy for historical and dispersal-related processes: distant communities can evolve in isolation from each other, and dispersal from patch to patch may be limited for individual taxa. In addition, environmental parameters can determine which taxa can or cannot survive or compete in a habitat patch. It is often difficult to distinguish spatial from environmental processes in the field because environmental differences correlate with distance.

Correlations among species composition, geographic distance, and environmental factors suggest that environmental processes structure pitcher fungal communities more than spatial processes do. Decay in assemblage similarity over space was driven by whether or not two plants were found in the same location, not distance from one plant to another. In addition, when we controlled for distance, temperature explained a large degree of variance in community similarity. We infer that decay in community similarity over distance is the result of local environmental conditions. The patterns we observe are congruent with patterns previously observed for endophytic and endolichenic fungi. U'Ren *et al.* (2012) concluded that climate and host type structured endophytic and endolichenic communities more than geography. Although both Hoffman & Arnold (2008) and Davis & Shaw (2008) found that geographic location explained endophyte community differences, neither explicitly documented environmental factors besides host identity and host phylogeny. We consider our analyses preliminary because we sampled few sites, our sites were on a latitudinal gradient where distance among sites correlates closely with difference in latitude (Mantel  $r=0.85$ ,  $p=0.001$ , 999 permutations), and we used coarse-scale temperature and precipitation data. Future observations must focus on

local environmental factors and on environmental factors that might correlate with temperature or location, including substrate pH, nutrient concentrations, sunlight, or the presence of other pitcher-inhabiting organisms. In addition, experimental work specifically investigating metacommunity processes, including dispersal and competition, will complement our understanding of diversity over space.

Fungal communities differentiate over broader spatial scales than pitcher plant bacterial communities do; we observed distinct fungal communities over thousands of kilometers, but other researchers observed distinct bacterial communities over tens to hundreds of kilometers. Peterson *et al.* (2008) assayed *S. purpurea* bacteria using tRFLP in Massachusetts bogs, and found distinct bacterial communities in bogs 28 to 123 km apart. Koopman & Carstens (2011) assayed bacteria in pitchers of *S. alata*, a southern species of *Sarracenia*, using 454 sequencing, and found distinct communities in populations spaced between 10 and 310 km in the US state of Louisiana. We speculate that bacterial species are sensitive to finer-grained environmental conditions than fungal species are, and that environmental sensitivity drives both bacterial and fungal community composition. For example, Buckley *et al.* (2010) found that pitcher bacterial morphospecies composition correlates with local-scale environmental factors including pitcher age, pitcher shape, plant size, and sphagnum and tree cover, while we found that fungal OTU composition correlates with average yearly temperature, a regional-scale environmental factor.

Pitcher fungal community structure also correlated with population structure of *Wyeomyia smithii*, the pitcher plant mosquito. *Wyeomyia smithii* only lives in pitcher plants, and ranges throughout the range of *S. purpurea* and *S. rosea*. It is a top predator,

and controls the abundance and diversity of other organisms in the food web, including pitcher bacteria. For example, pitchers with greater numbers of *W. smithii* larvae had higher bacterial alpha diversity (Peterson *et al.* 2008, Kneitel & Miller 2002), but lower bacterial abundance (Hoekman 2007) than pitchers with smaller numbers of or no *W. smithii*. *Wyeomyia smithii* has two population groups: northern and southern, with a division between the two in North Carolina (Emerson *et al.* 2010). Fungal community composition is also divided between northern and southern locations (Figure 2.2a). We speculate that *W. smithii* may directly shape fungal communities, or that similar processes structure *W. smithii* populations and fungal communities. Fungi also potentially interact with their *Sarracenia* host and with other pitcher-inhabiting organisms, and many of the widespread sequences we observed are from symbiotic taxa (*e.g.*, *Lecanicillium*, an insect parasite genus; *Fusarium*, a plant pathogen genus).

Finally, fungi from the surrounding area may impact the assemblages we observe in pitchers. Fungal spores may arrive from forest or savanna that surrounds pitcher habitat, but not persist in pitchers. We consider this unlikely because subsets of our data set without Agaricomycete and unassigned fungi produced the same patterns as the full data set (Figure A1.3). By eliminating Agaricomycetes and unassigned fungi, we removed the taxa that we consider least likely to be true pitcher inhabitants and most likely to produce numerous spores outside of pitchers; we did not entirely eliminate the possibility that the community divisions we observed are the result of local spores.

The taxonomic identities of sequences give preliminary clues about fungal ecological functions in pitchers. For example, Ascomycetes were the most widespread fungi (Figure 2.4b). Widespread or frequently observed Ascomycete classes included the

Dothideomycetes, Leotiomyces, and Sordariomycetes. All three of these classes include saprobic (*e.g.*, *Nectria*, Kodsueb *et al.* 2008), plant pathogenic (*e.g.*, *Cladosporium*, Rivas & Thomas 2005), plant epiphytic, and plant endophytic (*e.g.*, *Aureobasidium*, Botella & Diez 2011) taxa. Notably, the Capnodiales are well represented in both data sets.

Capnodiales is a fungal order in the class Dothideomycetes best known for housing the sooty molds, epiphytic fungi that consume honeydew (Crous *et al.* 2009). *Sarracenia* pitchers produce nectar to attract prey insects (Bennett & Ellison 2009), and nectar is a possible food source for these and other fungi. In addition, Basidiomycete yeast taxa including *Rhodotorula*, *Sporobolomyces*, and *Cryptococcus* were widespread or frequently observed in our data sets: each of these genera occupied at least two sites.

Basidiomycete yeasts are often plant epiphytes that consume molecules diffused from intact or damaged leaf tissues including sugars, organic acids, and amino acids (Fonseca & Ignácio 2005). We also observed many insect associated taxa including *Cordyceps* and *Verticillium* (Evans 1982). Taxa such as these may associate with either pitcher-inhabiting insects (inquilines) or prey insects as mutualists or pathogens. Finally, we were not able to assign taxonomic identities to 28% of geographic samples and 29% of temporal samples. These sequences may be from fungi that have yet to be described or characterized.

#### *Fungal community patterns over time*

Fungal communities develop quickly early in pitchers' lifespans, and continue to develop slowly for at least two months after pitchers open. We observed distinct young (week old) and old (month and two-month old) fungal communities; month and two-

month communities were also slightly, but not significantly, different from each other. Pitchers are sterile before opening (Peterson *et al.* 2008). Once open, young pitchers contained the highest number of taxa, and taxon number declined over time.

Environmental changes within pitchers, differences in fungal dispersal over time, and interactions among organisms may shape fungal succession. Future observational and experimental work will tease these processes apart by tracking individual taxa over time and examining competitive interactions among taxa. Correlations among fungal community composition, pitcher chemistry, prey abundance, and inquiline abundance suggest hypotheses assigning ecological processes to observed patterns.

Available data on pitcher water pH correlate with our observations on fungal diversity over time, although we did not measure pitcher water pH for this study. Fish & Hall (1978) observed that *S. purpurea* pitcher fluid pH is least acidic when pitchers are ~8 days old, and is most acidic from an age of ~35 days until at least 90 days. We observed significantly different fungal communities at the least acidic (week timepoint) and most acidic (month and two-month timepoints) pitcher ages; fungi observed at these timepoints may differ in their optimum pH for growth.

Fungal diversity also correlates with insect capture rate. *Sarracenia purpurea* insect capture rate peaks when pitchers are between 10 and 20 days old (Fish & Hall 1978). We may have observed many fungi not transported by prey insect vectors at the week timepoint, and many fungi transported by prey insect vectors at the month and two-month timepoints. Other potential insect vectors that are not prey include *W. smithii*, which oviposits in pitchers immediately after pitchers open (Fish & Hall 1978, Miller & Kneitel 2005).

Changes in fungal assemblages over time may be the result of seasonal environmental changes. Koopman *et al.* (2010) observed that *Sarracenia flava* pitcher bacterial composition depends on the month in which the pitcher was collected; they did not explicitly examine pitcher age. We did not control for season in our sampling (*i.e.*, all month old samples were collected in July; there were no month old samples collected in June or August, although month-old pitchers did exist in the field at these times), and so the changes we observed in fungal diversity may have reflected seasonal changes instead of or in addition to pitcher successional changes. Also, Koopman *et al.* observed that bacterial diversity increased over the first four months of the growing season, which contrasts with our observations of decreasing fungal diversity over the first two months of the growing season.

Dispersal may interact with competition to shape pitcher fungal diversity. Competition shapes fungal succession in other ephemeral habitats; for example, late-successional fungi are superior competitors to early-successional fungi on decomposing wood (Holmer & Stenlid 1997). Both antagonistic and resource competition shape fungal communities in cheese (reviewed in Irlinger & Mounier 2009), and competition interacts with dispersal by insect vectors in yeast communities on Amazon fruit (Morias *et al.* 1995). Dispersal and competition often trade off with each other in ephemeral habitats (Leibold *et al.* 2004); we may have observed good fungal dispersers early in succession that were then outcompeted by good competitors late in succession.

## Conclusions

Pitcher plant fungal communities are diverse: although we did not saturate our sampling curves, we observed tens of taxa at each location or timepoint, and hundreds of taxa in each data set. We observed changes in communities over both space and time. Correlations between community structure and environmental parameters suggest that both abiotic (temperature, pH, and correlated parameters) and biotic (interactions with *W. smithii* and insect prey including potential insect vectors) interactions shape fungal species diversity. Future observational and experimental work will identify the specific processes leading to observed patterns, and the relative influences of space and time on fungal diversity in pitchers.



## **CHAPTER 3**

### **Dispersal and competition structure microbial metacommunities in pitcher plants**

## **Abstract**

Dispersal among communities is a key driver of metcommunity diversity. Microbial dispersal may be homogeneous within a metacommunity, or different species may disperse in greater or lesser numbers, at different times, or both. Here we report substantial differences in the timing of yeast species' dispersals into carnivorous plant pitchers. We used a molecular assay to directly observe yeast dispersal into pitchers over two months, and found that three yeast species arrived in pitchers sequentially. In addition, we artificially manipulated yeast dispersal into natural pitchers and laboratory microcosms to test whether dispersal or environmental changes in pitchers led to the patterns observed in nature, and to understand how dispersal differences can alter competitive outcomes among yeasts. Appearances of yeasts in pitchers at different times in the growing season reflected dispersal, not changes in the pitcher environment over successional time. In microcosms, the three investigated yeast species were organized in a competitive hierarchy, but dispersal of greater numbers of propagules gave each yeast a competitive advantage. Our data show that different yeast species have different temporal patterns of dispersal and that dispersal limitation, contingency, and interactions between dispersal and competition can shape microbial diversity in the field.

## Introduction

Dispersal can link local communities into larger metacommunities (Leibold *et al.* 2004). Metacommunities are groups of incompletely isolated communities: community processes like competition occur within each community, and individual communities are imperfectly linked through dispersal. Microbial metacommunities are ubiquitous, and include bacteria in rock pools (Langenheder *et al.* 2012), mycorrhizal fungi occupying tree “islands” (Peay *et al.* 2007), and bacteria in the lungs of cystic fibrosis patients (Van der Gast *et al.* 2011). Although dispersal appears to be a critical control of microbial diversity in communities and metacommunities (Martiny *et al.* 2006), the dynamics of microbial dispersal in nature remain poorly studied: we lack direct observations of microbial dispersal, especially over time.

The temporal dispersal of microbes in nature may range from saturating to limiting. In a group of discrete habitats, species with saturating dispersal will reach all habitats immediately after the habitats become available. Saturating dispersal can decrease species diversity by allowing good competitors to swamp out poor competitors (Kerr *et al.* 2002). If dispersal is saturating for every species in a spatially structured community, the species compositions of individual patches may not diverge; despite their spatial structure, such communities are single communities and not true metacommunities because individual patches have the same species composition as the broader community (Holyoak *et al.* 2005). At the other extreme, if every species in a metacommunity is dispersal limited, many otherwise suitable habitats will remain uncolonized for some length of time before a microbial species arrives. Dispersal

limitation can also reduce population resilience to local extinctions (Brown & Kodrick-Brown 1977).

Different species may experience different degrees of saturation or dispersal limitation: for example, different species may colonize habitats at different times throughout each habitat's history. Differences in the timing of dispersal are thought to influence metacommunity diversity as each local assemblage is influenced by tradeoffs between competitive and dispersal abilities (Leibold *et al.* 2004; Kennedy & Bruns 2005). In contrast, metacommunities composed of species dispersing simultaneously are more likely to be shaped directly or exclusively by local deterministic processes or ecological drift.

Microbial ecologists have challenged the long-standing assumption that dispersal has little influence on global microbial diversity (*e.g.*, Whitaker *et al.* 2003), but previous research on microbial dispersal has focused on spatial patterns of diversity, not temporal differences in dispersal (*e.g.*, Van der Gucht *et al.* 2007). Microbial succession, especially of biofilms, is well documented, but changes in biofilm communities over time may be caused by temporal differences in dispersal or environmental changes in the biofilm itself (*e.g.*, Burmølle *et al.* 2007; Dang *et al.* 2008), and current research cannot distinguish between these two mechanisms.

We used yeasts inhabiting pitchers of the carnivorous plant *Sarracenia purpurea* as a model for experiments and observations to examine microbial dispersal and the influence of dispersal on competition among yeast species. This plant produces modified leaves, or pitchers (Figure 3.1), that fill with rainwater. Potential prey (ants and other small insects) are attracted to pitchers (Bennett & Ellison 2009); some of them fall into



*Figure 3.1: An example of Sarracenia purpurea with pitchers used in this study. Note the appearance of opened (exposed) and closed (sterile) pitchers.*

pitchers and drown, and are then shredded, decomposed, and mineralized by a food web of microorganisms and invertebrates (reviewed in Ellison *et al.* 2003). *Sarracenia purpurea* hosts a number of yeast (single-celled fungal) species, some of which are easy to culture. A population of pitcher plants whose pitchers contain yeasts can form a metacommunity: if dispersal is not saturating for all yeast species, each pitcher will house a community of yeasts that is incompletely isolated from other pitchers. We hypothesize that yeasts disperse among pitchers with some dispersal limitation and that a population of pitcher plants houses a yeast metacommunity. The functions of yeasts within the pitchers are not clear, although bacteria appear to form the base of the invertebrate food web (Kneitel & Miller 2002). Pitchers are sterile before opening and therefore yeasts cannot colonize a pitcher until after it has opened (Peterson *et al.* 2008). We isolated three easily manipulated pitcher plant yeasts and developed molecular tools to identify and track these species in nature.

To confirm this system as a metacommunity, describe temporal dynamics of dispersal in nature, test whether microbial species experience different degrees of dispersal limitation, and understand how dispersal influences competition, we asked three questions: 1) Do yeasts appear in pitchers as soon as the pitchers open, and do all yeast species appear at the same time? 2) Are temporally different appearances due to different dispersal times or to successional changes within the pitcher habitat? 3) Can differences in dispersal among yeasts alter outcomes of yeast competition within pitchers? We used field observations, a field experiment, and laboratory microcosms to answer these questions.

## Materials and Methods

### *Study site*

Observations were made and the field experiment was conducted on isolated *Sphagnum* islands in Harvard Pond, adjacent to Tom Swamp, a 50 ha *Sphagnum* bog located in Petersham, Massachusetts at 42°30'N, 72°12'W (Figure 3.2a; Swan & Gill 2007). The yeasts used in this study were collected from pitcher plants growing on these bog islands and at Swift River Bog, a 2 ha kettlehole bog located 75 km south of Tom Swamp in Belchertown, MA at 42°16'N, 72°20'W (Ellison *et al.* 2002).

### *Yeast isolation and identification*

We collected yeast isolates from pitchers during the summer of 2006. We chose three target species that grow as morphologically distinct colonies, facilitating differentiation on Petri dishes. *Candida glabrata* (which produces smooth white colonies) and *Pseudozyma aphidis* (wavy white colonies) were collected from Harvard Pond; *Rhodotorula glutinis* (smooth pink colonies) was collected from Swift River Bog. To isolate yeasts, water was collected from the inside of pitchers using sterile transfer pipettes, diluted, and plated onto solid media (1g/L yeast extract in tap water with 1.5% agar; 50 µg/ml streptomycin, penicillin, and ampicillin were added to prevent bacterial growth). Individual yeast colonies were streaked onto fresh plates when they became visible. We sequenced two ribosomal sequences for each isolate using the primer pairs ITS1F/ITS4 (Gardes & Bruns 1993, White *et al.* 1990) and LS1/LR5 (Hausner *et al.* 1993, Vilgalys & Hester 1990, see PCR assay methods below). Sequences were identified to species using the NCBI BLAST database (Zhang *et al.* 2000, Table A2.1).

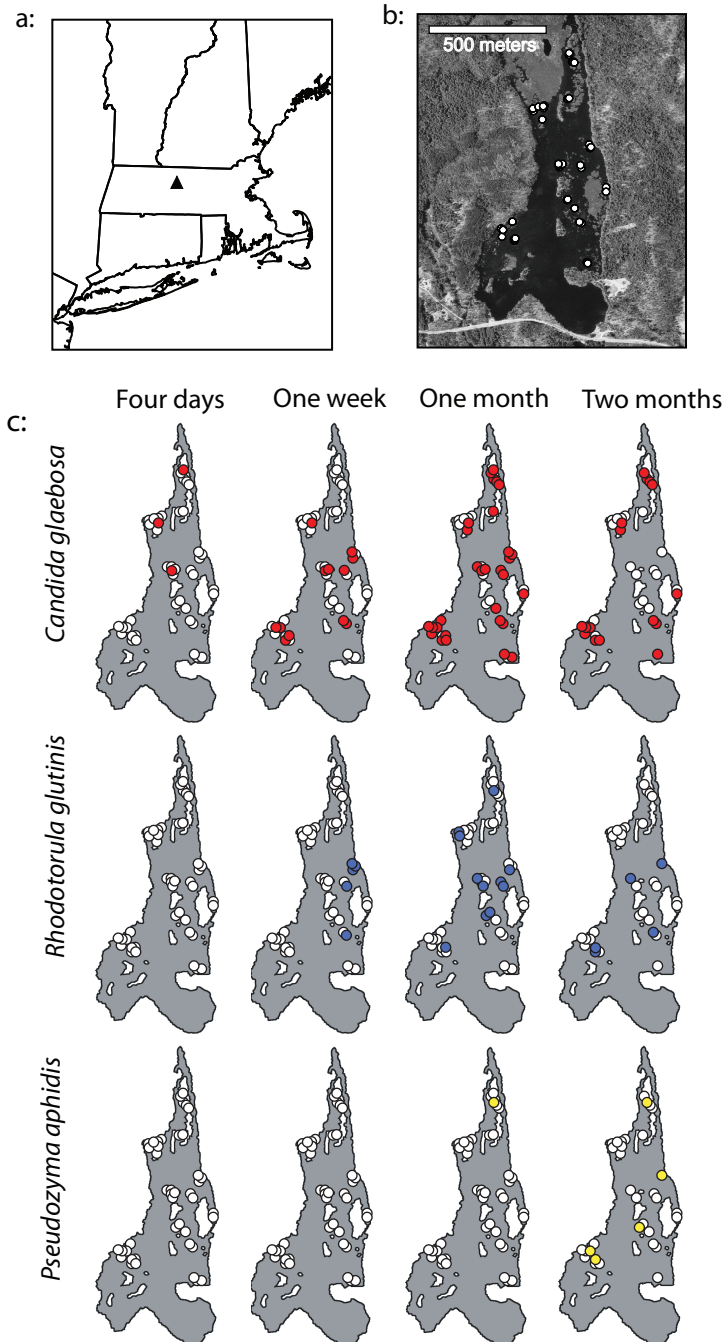


Figure 3.2: Locations of bog and pitchers, and presences of each yeast in pitchers. 3.2a: Location of Tom Swamp and Harvard Pond in Petersham, Massachusetts. 3.2b: Locations of pitchers sampled, indicated using white dots. Note that some dots are obscured by nearby dots and are not visible on this map. 3.2c: Yeast arrivals in pitchers over two months. Colored dots represent presences and white dots absences. Some dots have been shifted slightly to make the data for all pitchers visible. Red = *Candida glabrata*, blue = *Rhodotorula glutinis*, yellow = *Pseudozyma aphidis*. Maps created using ArcMap™ Version 9.2 (ESRI, 2006); map data from the Office of Geographic Information, Commonwealth of Massachusetts Information Technology Division (2000) and the National Atlas of the United States (2006).



### *Field collections*

In May of 2009, we identified 43 unopened *S. purpurea* pitchers on 32 *Sphagnum* islands in Harvard Pond (Figure 3.2b) and recorded the location and opening date of each pitcher. Pitchers ranged from less than 1 m to 908 m in distance to other pitchers. We collected water from each pitcher 4, 6-9 (hereafter referred to as one week), and 34-42 days (one month) after it opened. We also collected water from pitchers after 66-74 days (two months), although by then insect herbivores, including moth larvae (Atwater *et al.* 2006), had destroyed ten of the original 43 pitchers, and we could only sample water at this last date from 33 pitchers. We collected water from the bog itself within 0.5 m of each of 17 of the pitchers at the one-month timepoint to determine whether target species were also found in bog water. For each pitcher water collection, the water inside a pitcher was mixed by pipetting up and down with a sterile plastic transfer pipette. We removed about 0.25 ml pitcher water and mixed it with 0.25 ml CTAB buffer. To the best of our ability, we avoided collecting insect prey or macrofauna in these samples, although any protists and microscopic animals present in our samples were included; collected pitcher water contained no large animal parts and appeared as a cloudy liquid. Bog water samples consisted of 0.25 ml of water from just below the bog surface collected using a sterile transfer pipette and mixed with 0.25 ml CTAB buffer. All samples were flash-frozen in liquid nitrogen within five hours of collection and stored at -20 or -80°C.

### *Primer design and PCR assay*

Primers to selectively amplify portions of the Internal Transcribed Spacer (ITS) sequence and uniquely identify each of the three yeasts were designed using the NCBI

BLAST primer tool (Rozen & Skaletsky 2000; Table 3.1). We chose primer sequences to reliably amplify as much of the ITS sequence of each yeast species as possible, while not amplifying other sequences in the BLAST database. We tested each primer pair for sensitivity by 1) counting the number of colony-forming units (CFUs) of each yeast in five liquid cultures using plate counts and calculating the concentration of CFUs in each liquid culture; 2) extracting genomic DNA from each liquid culture; 3) amplifying serial dilutions of DNA extracts (see PCR conditions below); and 4) back-calculating the minimum numbers of CFUs detected based on the most dilute DNA extract that produced a detectable PCR product. Primers amplified DNA from a minimum of  $159.4 \pm 183.0$  cells/ml (mean  $\pm$  standard deviation) of *C. glabrosa*,  $39.12 \pm 22.0$  cells/ml of *R. glutinis*, and  $43.9 \pm 45.1$  cells/ml of *P. aphidis* (n=5).

Table 3.1: Sequences of primers used to detect presence of each yeast in a pitcher.

yeast	primer	forward sequence	Tm	product length
<i>C. glabrosa</i>	P21-17-F	5'-CTGCGGAAGGATCATTACAGT-3'	54.6	
<i>C. glabrosa</i>	P21-17-R	5'-TGTTTCAGACAACACTGTTCA-3'	51.8	466
<i>R. glutinis</i>	B11-4-F	5'-AAGTCGTAACAAGGTTTCCG-3'	52.8	
<i>R. glutinis</i>	B11-4-R	5'-CCCAACTCGGCTCTAGTAAA-3'	53.9	527
<i>P. aphidis</i>	46A-3-F	5'-GGTAATGCGGTCGTCTAAAA-3'	52.6	
<i>P. aphidis</i>	46A-3-R	5'-CTCTTCCAAAGAAGCGAGG-3'	53.1	467

We tested each extract for the presence of each target yeast using the three yeast-specific primer pairs. We also tested each extract for the presence of any amplifiable fungi using the ITS1F/ITS4 primer pair. DNA was extracted from samples as described in Chapter 2. Each PCR reaction was composed of 7.9  $\mu$ L of water, 0.1  $\mu$ L of GoTaq® polymerase (Promega), 5  $\mu$ L of flexi buffer with green dye added, 5  $\mu$ L of CES (Ralser *et al.* 2006), 5  $\mu$ L of nucleotide mix, 2  $\mu$ L of magnesium chloride, 1  $\mu$ L of 10  $\mu$ M of each primer, and 1  $\mu$ L of template DNA extract. All reactions were cycled on a Biorad iCycler

or myCycler using denaturing, annealing, and extension temperatures of 95, 55, and 72 °C, respectively. We denatured for 85 s, then ran 13 cycles of 35 s denaturing, 55 s annealing, and 45 s extension, followed by 13 cycles that were identical but had a 2 min extension, and finally 9 cycles with a 3 min extension. We ran a subsequent 10 min extension. Two µL of each PCR product were visualized on 1% agarose gels stained with SYBR® Safe dye (Invitrogen) and photographed using a U:genius gel documenting system and a Stratagene transilluminator. Photographs of gels were scored for presence or absence of a band. Bands that were too faint to reliably score were run a second time with 6 µL of PCR product per well. Presence of a band on a gel indicated presence of that yeast (or of some detectable fungal DNA, in the case of the ITS1F/ITS4 primer pair) in a water sample.

To confirm that primers only amplified sequences from the target yeasts, we randomly selected nine PCR products generated from the *C. glabrosa* and *R. glutinis* primer pairs for sequencing. The primer pair that targets *P. aphidis* only amplified DNA from seven pitcher water extracts, and we sequenced all seven PCR products for this primer pair. Sequences were identical to or within one base of the reference sequences.

#### *Survival of P. aphidis in young pitchers and subsequent colonization observations*

To test whether the latest arriving yeast (*P. aphidis*) could survive in young pitchers, we inoculated it into newly opened pitchers the following year (late May, 2010), and monitored its subsequent survival. Survival of *P. aphidis* was compared with that of *C. glabrosa*, the earliest arriving yeast, and with uninoculated controls. We also monitored control pitchers for natural arrivals of both *P. aphidis* and *C. glabrosa*. To

achieve these goals, we selected 45 pitchers on bog islands at Harvard Pond that were either closed but mature or just barely open, and we manually opened closed pitchers. Once opened, we randomly assigned each pitcher to one of the following three treatments: a stationary phase liquid culture of *P. aphidis* ( $1.3 \times 10^6$  cells total); a stationary phase liquid culture of *C. glabrosa* ( $1.9 \times 10^5$  cells total); or filter-sterilized media on which *P. aphidis* had previously reached stationary phase (“control pitchers”). Because the goal of this experiment was to compare the latest colonizer *P. aphidis* to the earliest colonizer *C. glabrosa*, *R. glutinis* was not inoculated into any treatments in this experiment. In total, we added 200  $\mu$ L of liquid to each pitcher, and we inoculated 15 pitchers for each treatment. After 4 and 22 days, water was removed from each surviving pitcher that contained rainwater (13 pitchers for each treatment after 4 days; 15 pitchers for *C. glabrosa* and control pitchers and 15 pitchers for *P. aphidis* after 22 days) and checked for the presence of each of the 2 inoculated yeasts using the methods described above.

### *Competition assays*

To test whether interactions between dispersal and competition affect yeast coexistence, we prepared laboratory microcosms in which pairs of the three isolates competed. Microcosms mimicked pitchers in the field that were hypothetically colonized by different numbers of propagules of two yeast species. Each microcosm contained a target species and a competitor in 200  $\mu$ L of liquid media (1 g yeast extract/1 L tap water). Each target species was inoculated with  $\sim 1000$  cells per microcosm (medium

Table 3.2: Numbers of cells (with standard deviations) inoculated into microcosms.

yeast	low	medium	high
<i>C. glabrosa</i>	64.8±9.8	647.8±98.0	6477.8±979.5
<i>R. glutinis</i>	55.2±8.6	552±85.7	5520±857.3
<i>P. aphidis</i>	126.7±22.1	1266.7±221.4	12666.7±2214.2

inoculation), while each competitor was inoculated at zero, low, medium, and high inoculations (0 and ~100, 1000, and 10 000 cells; actual numbers of cells in Table 3.2).

Eighteen treatments of yeast mixtures were prepared, with 10 replicates each, for a total of 180 microcosms on 7 cell culture plates. Before being used to inoculate microcosms, yeasts were grown in liquid media for 48 hours to stationary phase. Optical density was used to estimate cell number, and cultures were diluted to inoculation concentrations using liquid media. Microcosms were arranged in sterile 96-well polystyrene flat bottom cell culture plates. As a control for splashing, we put 200 µL of uninoculated media into each edge well and every second central well. Treatments were randomized to the remaining wells (thirty remaining wells per plate). Microcosms neighboring uninoculated wells that showed signs of contamination by the end of the experiment were discarded. Microcosms were incubated between 25 and 27 °C, shaking at 700 rpm for 48 hours before assaying. At the same time that we prepared microcosms, we also prepared ten 100 × 15 mm Petri dishes of solid media (1 g yeast extract/1 L tap water; 1.5% agar added) for each yeast to determine precise numbers of CFUs inoculated. These were incubated for five days at room temperature before colonies were counted.

After 48 hours of incubation, each non-discarded microcosm (148 out of 180 microcosms) was diluted 1:10<sup>3</sup> and 1:10<sup>4</sup> in sterile tap water. Fifty µL of each diluted microcosm were spread onto a 100 × 15 mm Petri dish containing solid media. Dishes were incubated at room temperature, and colonies counted after five days.

### *Statistical analyses*

We tested spatial clustering of each yeast within the bog at each timepoint using Mantel tests to identify correlations between the presence of each yeast and location. Mantel tests were conducted in R version 2.6.1 using the vegan library version 1.11-4 (R Development Core Team 2007; Oksanen *et al.* 2008).

We explored the impact of competitor inoculum size on the growth of each yeast using linear regressions, also using R. For each yeast, we produced two regressions, one for its response to each of its competitors. The dependent variable for these regressions was relative yield, defined as the number of cells produced in the presence of the competitor divided by the average number of cells produced in controls (Harper 1977); the independent variable was number of competitor cells added. For each yeast, differences between the two competitor regressions were tested using a two-slope t-test and an ANCOVA for identical regression slopes and intercepts, respectively. The two competitors of *P. aphidis* produced regressions with the same slope and intercept, and so we produced a single regression for this target species using the response of *P. aphidis* to both competitors combined (Table A2.2).

## **Results**

*Time, not space, correlates with the order of yeast colonizations into *S. purpurea* pitchers*

The yeasts *C. glabrosa*, *R. glutinis*, and *P. aphidis* appeared in pitchers sequentially. *Candida glabrosa* appeared in *S. purpurea* pitchers within four days after the pitchers opened; *R. glutinis* arrived between four days and one week after pitchers

opened; and *P. aphidis* arrived one week to one month after pitchers opened (Figure 3.2c). Once each yeast had colonized a pitcher, it either persisted in or disappeared from that pitcher later in the season, but it did not disappear from the broader metacommunity. Fungal DNA was detectable using fungal specific primers (ITS1F/ITS4) starting from the first measured timepoint (in 33% of sampled plants), and was widespread after one week, one month, and two months (in 91%, 95%, and 73% of sampled plants, respectively). Spatial clustering of colonization was not evident (no significant spatial clustering by Mantel test; Table A2.3). None of the bog water samples contained *C. glabrosa*, *R. glutinis*, or *P. aphidis*, although 59% of the 17 samples contained fungal DNA detectable with the ITS1F/ITS4 primer pair.

#### *A late-arriving yeast survives in early successional pitchers*

We detected the inoculated yeasts *P. aphidis* and *C. glabrosa* in 100% of their respective inoculated pitchers containing rainwater after 4 and 22 days (pitchers not containing rainwater were not investigated). In addition, 9 control pitchers contained *C. glabrosa* and 0 contained *P. aphidis* after 4 days, whereas 14 control pitchers contained *C. glabrosa* and 7 contained *P. aphidis* after 22 days, confirming patterns observed the previous year.

#### *Dispersal differences alter yeast competitive outcomes*

In each yeast-competitor pairing, large competitor inoculum sizes decreased the growth of each target yeast (Table 3.3, Figure 3.3). Only *R. glutinis*, when challenged with *C. glabrosa*, experienced a negative effect at low competitor inoculum sizes

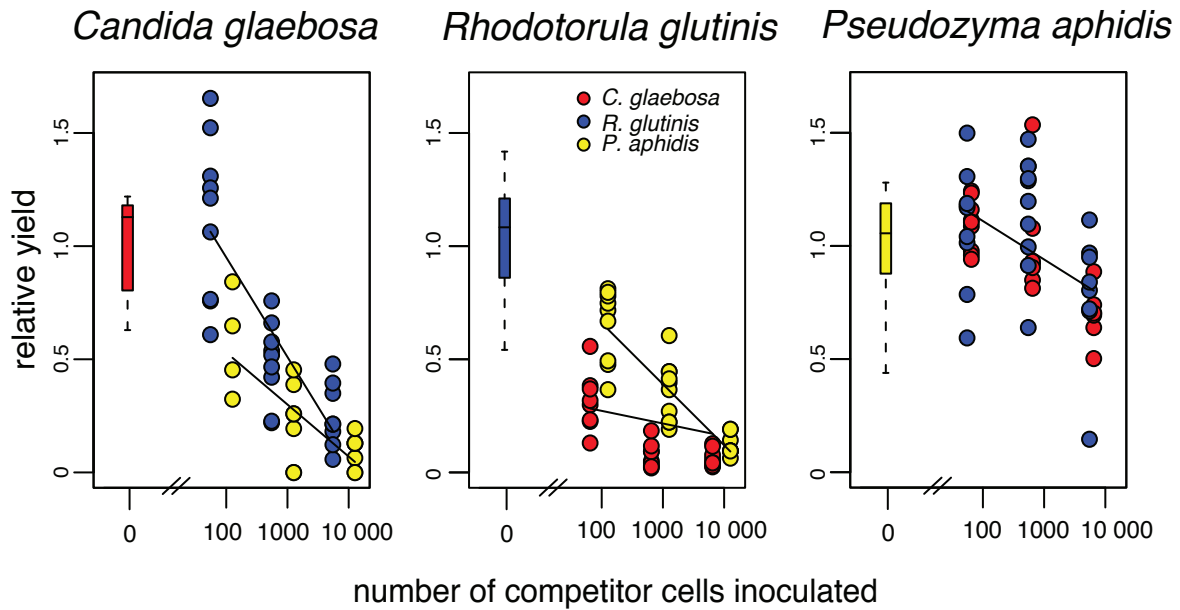


Figure 3.3: Impact of competitor inoculum size on cells produced for each studied yeast. Points and regressions indicate the relative yield (Harper 1977) of each yeast (relative yield = cells produced in the presence of a competitor / cells produced in controls) as a function of the numbers of cells of each competitor inoculated. Each panel indicates the relative yield of a single target yeast whose inoculum size was held constant. Points give the relative yield of the target yeast in the presence of each possible competitor. Box-and-whisker plots give the ranges, first quartile, median, and third quartile of control values. By definition, the means of the control values are equal to one. Red = *Candida glabrosa*, blue = *Rhodotorula glutinis*, yellow = *Pseudozyma aphidis*.



(y-intercept of 0.50, but y-intercept  $\geq 0.99$  for every other pairing). The two possible competitors of each of *C. glabrosa* and *R. glutinis* decreased the relative yields of these yeasts to different extents, but the two possible competitors of *P. aphidis* decreased its relative yield by the same amount. In the face of competition, relative yield of *P. aphidis* also decreased less than that of other yeasts. In addition, competitor inoculum size explained less variation in *P. aphidis* growth than in any other pairing (adjusted  $r^2=0.24$ ; Table 3.3). In general, the presence of competitors reduced growth of heterospecific yeasts, and larger inoculations of competitors inhibited heterospecifics more than smaller inoculations did. Competitive ability calculated for equal inoculum sizes shows a competitive hierarchy of *P. aphidis* outcompeting *C. glabrosa*, and both of these outcompeting *R. glutinis* (Table 3.4).

Table 3.3: regression equations and statistics for regressions of relative yield of each target yeast (relative yield = cells produced in the presence of a competitor / cells produced in the averages of controls; Harper, 1977) dependent on  $\log_{10}$  (competitor inoculum size).

target yeast	competitor	y-intercept	slope	adj $r^2$	$p$
<i>C. glabrosa</i>	<i>R. glutinis</i>	1.83	-0.44	0.65	$3.96 \times 10^{-7}$
<i>C. glabrosa</i>	<i>P. aphidis</i>	0.99	-0.23	0.53	0.0004
<i>R. glutinis</i>	<i>C. glabrosa</i>	0.50	-0.12	0.52	$4.55 \times 10^{-5}$
<i>R. glutinis</i>	<i>P. aphidis</i>	1.20	-0.27	0.75	$4.19 \times 10^{-9}$
<i>P. aphidis</i>	both	1.45	-0.17	0.24	0.0002

Table 3.4: calculated relative yields of each yeast with 1:1 dispersal with each possible competitor

species 1	species 2	relative yield (sp.1)	95% CI (sp. 1)	relative yield (sp. 2)	95% CI (sp. 2)
<i>P. aphidis</i>	<i>C. glabrosa</i>	0.922	0.846-0.997	0.352	0.258-0.446
<i>C. glabrosa</i>	<i>R. babjevae</i>	0.589	0.481-0.697	0.157	0.115-0.198
<i>P. aphidis</i>	<i>R. babjevae</i>	0.922	0.846-0.997	0.472	0.418-0.525

## Discussion

To our knowledge, ours are the first direct observations of different degrees of microbial dispersal limitation in a natural system. Because yeast dispersal into pitchers is unequal, and because pitchers did not all contain the same yeasts at any timepoint, we confirm that this system is a metacommunity. Previous researchers have found indirect evidence of different dispersal abilities of fungi and bacteria, based on colonization and spatial patterns of ectomycorrhizal fungi, temporal variation in lichen diversity, and variation in concentrations of airborne microorganisms (Kennedy & Bruns 2005; Peay *et al.* 2007; Caruso *et al.* 2010; Fierer *et al.* 2008). Others have demonstrated the differential dispersal of protists in artificially assembled laboratory microcosms (Cadotte *et al.* 2006) and succession in microbial biofilms (*e.g.*, Burmølle *et al.* 2007; Dang *et al.* 2008). However, it is not clear whether temporal differences in biofilm assemblages are due to different arrival times or to successional changes caused by biofilm development itself. Our data extend these studies by demonstrating that temporal patterns of microbial appearances can represent differences in the timing of dispersal. Although an analogous study in tree-hole assemblages has suggested that temporal differences in dispersal have only a minor influence on microbial communities (Bell 2010), our data and those cited above suggest that in fact there are widespread differences in dispersal abilities among microbes over time spans of weeks to months.

*Sarracenia* yeasts appear in pitcher habitats at different times; the three yeasts that we studied appear to disperse into pitchers sequentially. There are two alternative hypotheses that can explain the temporal sequence of appearance of these yeasts. First, the observed temporal sequence of yeast appearances could be due to successional and

environmental changes in the pitcher plant itself. The survival of *P. aphidis* (a late-arriving yeast) in young pitchers does not support this latter hypothesis. Alternatively, distance to occupied pitchers could explain the temporal sequence, with nearby yeasts arriving before distant ones. However, we found no evidence of dispersal (distance) limitation at a 50-ha spatial scale, suggesting that dispersal through time was more limiting than dispersal over space on the temporal and spatial scales of one season and one bog. Finally, a supplementary hypothesis is that interactions among yeasts (*e.g.*, competition) can sort out species once they arrive. We hypothesize that these interactions may enhance a pattern already produced by differently timed dispersals. The results of our microcosm experiment indeed suggest that large numbers of propagules of an initial colonist give it a potential competitive advantage in a given pitcher.

How do yeasts get to pitchers in the first place? Mechanisms of yeast dispersal into pitchers remain unclear. Yeasts are ubiquitous in nature (Kurtzman & Fell 1998), and potential sources of yeast inocula include: rainfall, older pitchers, the surfaces of other bog plants, surface or pore water of the bog itself, and surrounding forests and upland soils. Of these, our observations can only eliminate colonization directly from bog water splashing, because the surrounding bog water did not harbor the three yeasts we studied. Future work should explore spore deposition from air (Amend *et al.* 2010b) and transmission by arthropod prey or pitcher plant inquilines (Ellison *et al.* 2003; Gilbert 1980). Different numbers of propagules produced by different yeasts, different population sizes in the surrounding matrix, interactions with insect vectors, and additional processes may also contribute to yeast dispersal.

*Interactions between dispersal and within-pitcher processes alter metacommunity composition*

In microcosms, greater dispersal gave each yeast a competitive advantage, and different isolates had different competitive abilities. Because we do not yet know how yeasts disperse into pitchers, our inoculations into microcosms could represent one of two scenarios in the field: larger numbers of one species might arrive at the same time as smaller numbers of a competitor or early dispersers might grow rapidly and pre-empt later ones. In either case, our results suggest that a prolific or early disperser will have a competitive advantage. The variation in competitive and dispersal abilities that we observed also suggest that competition-dispersal tradeoffs exist in yeasts in nature, with early arriving yeasts gaining a temporary competitive advantage. Additional support for the existence of competition-dispersal tradeoffs in the *Sarracenia* system is provided by *P. aphidis*, which was the last yeast we observed in pitchers and was also the best competitor. *Pseudozyma aphidis* was negligibly influenced by greater numbers of competitors, and we speculate that it may have experienced selection for the ability to colonize patches that are already occupied. The term “patch dynamics” is sometimes used to describe metacommunities where tradeoffs between competition and dispersal dominate (Leibold *et al.* 2004). Patch dynamics metacommunities are frequently found in ephemeral habitats like *Sarracenia* pitchers, where uncolonized patches sometimes come into existence, while occupied patches sometimes disappear.

## Conclusions

Dispersal shapes the biodiversity of metacommunities, together with other ecological processes. For example, good dispersers are frequently pioneer species in ecological succession, and can either inhibit or facilitate subsequent arrivals (Connell & Slatyer 1977). Moreover, priority effects often determine the species composition of a community (Kennedy & Bruns 2005; Fukami *et al.* 2010). Peay *et al.* (2012) recently showed that yeasts experience priority effects in nectar metacommunities, and that the strength of priority effects was correlated with phylogenetic relatedness. Such interactions are also possible in pitcher plant systems. Processes like succession and priority effects are contingent on dispersal: future research is needed to elucidate the role of variation in microbial dispersal abilities on contingent processes like these in the field.

*Sarracenia* yeasts appear in pitchers at different times during the growing season, and these appearances are the result of different arrival times, not successional facilitation. In addition, early dispersal appears to give individual yeasts a potential competitive advantage. Together, the interaction between different dispersal times and competitive abilities can result in tradeoffs, which may shape microbial diversity in *Sarracenia* pitchers. The interaction between dispersal and other metacommunity processes may also lead to the observed variation in species compositions among pitchers; yeasts in *Sarracenia* pitchers form a metacommunity and not a single community with saturating dispersal among patches.

Dispersal is not a homogeneous process in microbial communities. Instead, *Sarracenia* yeasts behave like many plant and animal groups (*e.g.*, Brunet & von Oheimb

1998). Not only do some microorganisms have smaller than global distributions (*e.g.*, James *et al.* 1999); they also experience dispersal limitation in different ways.

## **CHAPTER 4**

### **Population structure of a ubiquitous pitcher inhabiting fungus**

## Abstract

Different genotypes within a fungal species occupy different niches and perform different functions. To complement observations on fungal communities, we observed population structure in the most widespread and abundant pitcher fungus observed in Chapter 2, *Candia glabosa*, across the United States and Canada using AFLP. We observed three *C. glabosa* populations in five sampled locations, and *C. glabosa* population structure did not reflect fungal community structure. We propose processes that might shape *C. glabosa* population structure and contrast *C. glabosa* population and fungal community patterns and processes.



## Introduction

Enumerating fungal species identities across time and space gives an incomplete picture of biodiversity: genotypes within a species can vary in their spatial and temporal niches, and they can perform different functions in an ecosystem (reviewed for ectomycorrhizal fungi by Cairney 1999). For example, fungal decomposer assemblages with different genetic compositions have different rates of CO<sub>2</sub> evolution (Wilkinson *et al.* 2010). Here we describe population structure in a widespread pitcher plant fungus, and compare and contrast population structure with fungal community structure.

We isolated and genotyped colonies of *Candida glabrosa*, an Ascomycete yeast related to *Debaryomyces hansenii* and *Candida albicans* (Figure A3.1). *Candida glabrosa* dominates pitcher fungal communities: it was present and abundant at all locations and timepoints in our 454 study (Chapter 2), in which it comprised 42% of all sequences in the geographic data set and 42% of all sequences in the temporal data set. It was also present in 35 of 38 plants in the geographic data set and 45 of 51 pitcher-timepoint combinations in the temporal data set. Because of this abundance, we suspect that *C. glabrosa* heavily shapes ecological processes in pitchers. We genotyped *C. glabrosa* colonies using Amplified Fragment Length Polymorphism (AFLP, Vos *et al.* 1995). AFLP produces a genetic fingerprint of restriction site associated fragments of different lengths, and is frequently used to generate a large number of loci to infer population structure (*e.g.*, Herrera *et al.* 2011). We hypothesized that *C. glabrosa* population subdivision would reflect community divisions in the greater fungal community (described in Chapter 2) because the same processes often structure communities and populations (Vellend & Geber 2005).

## Materials and methods

We isolated *C. glabrosa* colonies for AFLP genotyping from an arbitrary subset of the pitchers sampled for 454 sequencing in Chapter 2 (Table 4.1). Sampling covered all populations described in Chapter 2. Pitcher water was diluted with sterile water and spread onto solid media (1 g/L yeast extract in sterile tap water with 1.5% agar; 50 µg/ml streptomycin, penicillin, and ampicillin were added to prevent bacterial growth).

Colonies resembling *C. glabrosa* in appearance (round and cream colored colonies) were streaked onto fresh plates. We confirmed the identities of isolates using the *C. glabrosa* specific PCR primers and protocol described in Chapter 3. After clustering AFLP genotypes into populations (see below), we chose sixteen isolates encompassing all studied genetic groups and locations, and confirmed the accuracy of the primer screen by sequencing their ITS regions using the ITS1F and ITS4 primer pair. All sequences were identical to or one base pair different from the *C. glabrosa* ITS sequence used to design the primer screen. In total, 87 isolates from 53 pitchers (28 plants) were isolated and genotyped using AFLP.

Table 4.1: Numbers of *C. glabrosa* isolates from each site.

Site	# <i>Candida glabrosa</i> isolates used for genotyping	# pitchers represented by <i>C. glabrosa</i> isolates
British Columbia	19	16
Newfoundland	16	9
Massachusetts	18	15
Georgia	7	6
Florida	17	11

### *AFLP analyses*

We performed AFLP fingerprinting of *C. glabrosa* isolates based on the protocols followed by Vos *et al.* (1995). First, we grew each isolate in liquid culture (1% yeast extract in sterile tap water), pelleted each culture, and extracted DNA as in Chapter 2. Then, we performed a restriction digest using EcoRI and MseI at the same time as a T4 ligation reaction (all enzymes from New England Biolabs). Each restriction-ligation reaction was composed of 50 ng of genomic DNA combined with 1x T4 ligase buffer, 1 mg/ml bovine serum albumin, 0.05 M sodium chloride, 0.6 U EcoRI, 0.6 U MseI, 12 U T4 DNA ligase, and 0.9  $\mu$ M of each EcoRI and MseI adaptor (Vos *et al.* 1995), for a total of 6.6  $\mu$ l. Restriction-ligation reactions were incubated at 37 °C for 2 hours.

Next, we performed the first (preselective) PCR amplification of fragments on each restriction-ligation product diluted 1:10 in water. Each preselective amplification was composed of 9  $\mu$ l AFLP Core mix (Applied Biosystems), 0.3  $\mu$ l of 10  $\mu$ M of each EcoRI+0 and MseI+0 primer (no selective nucleotides were added to preselective primers), and 2.4  $\mu$ l dilute restriction-ligation product. Preselective amplification reactions were cycled as follows: 72 °C for 2 min; 20 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min; and a final extension of 60 °C for 30 min.

We then diluted each preselective amplification product 1:20 in water and performed selective amplifications on each dilute product. We used four sets of selective primers: EcoRI+TC/MseI+CT, EcoRI+TG/MseI+CT, EcoRI+TG/MseI+CC, and EcoRI+AT/MseI+CT. Each EcoRI selective primer was labeled with the FAM fluorophore. Each selective amplification was composed of 7.5  $\mu$ l of AFLP Core mix, 0.5  $\mu$ l of 1  $\mu$ M of the EcoRI primer, 0.5  $\mu$ l of 5  $\mu$ M of the MseI primer, and 1.5  $\mu$ l of the

dilute preselective amplification product. Selective amplifications were cycled as follows: 94 °C for 2 min; 30 cycles of 94 °C for 20 s, the annealing temperature for 30 s, 72 °C for 2 min; and a final extension of 60 °C for 30 min. The annealing temperature was 66 °C at the first cycle, and decreased by 1 °C each cycle for 10 cycles. It remained at 56 °C for the remaining 20 cycles.

Selective amplification products were cleaned using Exosap-IT® (Affymetrix) according to the manufacturer's instructions, and run on an ABI 3730xl (Applied Biosystems) by Genewiz®, Inc. Chromatograms were visualized using Genemapper 4.0 (Applied Biosystems); peaks were first called by the software and then edited manually. We scored 72 total unambiguous, polymorphic peaks for the four primer pairs. We re-ran seven of the isolates from the restriction-ligation step to determine the error rate in peak calling. In re-run samples, 4.2% of base calls were different between runs.

### *Statistical analyses*

We conducted all analyses of *C. glabrosa* genotypes using a matrix of the presences and absences of each of the 72 scored peaks in each of 87 isolates. Because we do not know whether *C. glabrosa* is haploid or diploid, we conducted all analyses assuming both nuclear conditions. Analyses conducted assuming diploidy are reported in the text, and analyses conducted assuming haploidy, which did not differ qualitatively from diploid analyses, are reported in Appendix 3.

We visualized population structure by constructing a neighbor-joining tree on Jaccard dissimilarities between genotypes using the vegan and APE 3.0-3 packages in R (Paradis *et al.* 2004). Populations of *C. glabrosa* genotypes were also partitioned using

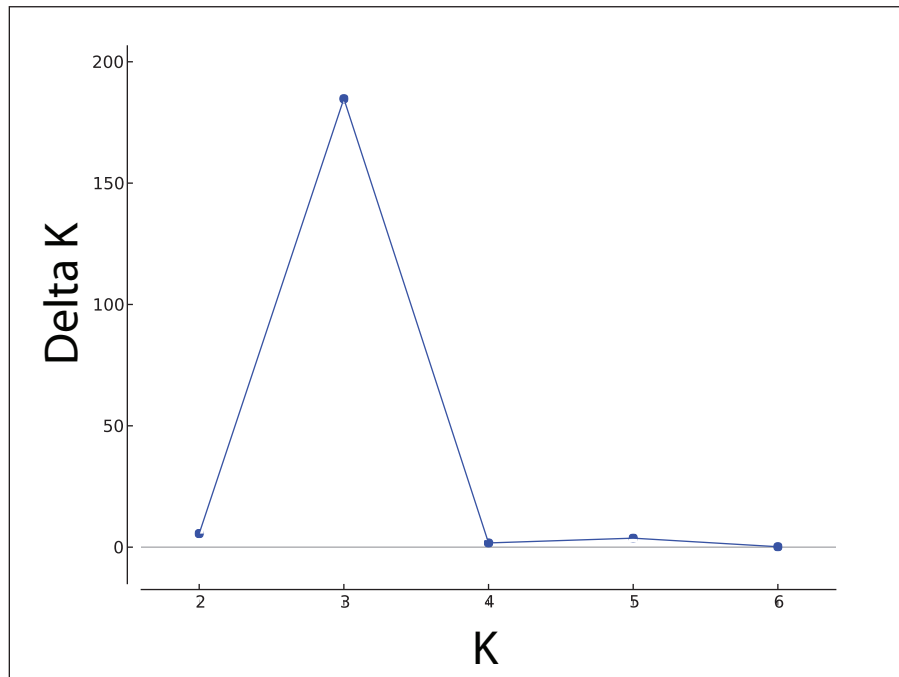
STRUCTURE 2.3.3 (Falush *et al.* 2007). We ran STRUCTURE for 10 replicates each of K values from 1 to 7 using a burn-in of 50 000 and MCMC of 100 000 cycles. We assumed admixture and independent allele frequencies among populations. We inferred the number of populations using Evanno *et al.*'s (2005) delta K and the program STRUCTURE HARVESTER (Earl & vonHoldt, 2012), and visualized STRUCTURE output using Distruct (Rosenberg 2004). One of the populations predicted by STRUCTURE and SRUCTURE HARVESTER contained isolates from three locations (Newfoundland, Massachusetts, and Georgia); we re-ran STRUCTURE analyses with each of these three locations removed to see if any one of them drove modeled population assignments.

We computed pairwise  $F_{st}$ s using AFLP-SURV (Vekemans *et al.* 2002). When assuming diploidy, we used the Bayesian method with uniform prior distribution of allele frequencies, and when assuming haploidy we used the method based on allele frequencies for estimating population and pairwise diversity.

## Results

A STRUCTURE analysis predicts three *C. glabrosa* populations: Evanno's delta K peaked at K = 3 (Figure 4.1a), and STRUCTURE assigned isolates to Florida, British Columbia, and eastern (Massachusetts, Newfoundland, and Georgia) populations (Figure 4.1b). When any of the three locations in the eastern population were removed from the analysis, STRUCTURE and STRUCTURE HARVESTER predicted the same three populations as for the full data set. A neighbor-joining tree of genetic similarities among all isolates also shows the same three genetic clusters (Figure 4.2). The Florida and

a:



b:

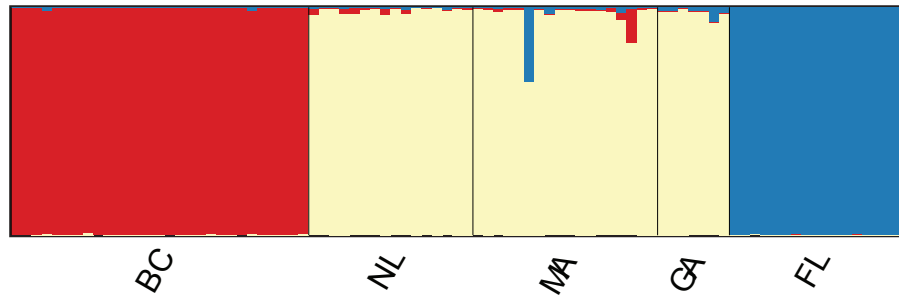


Figure 4.1: *Candida glabrosa* populations predicted by STRUCTURE. 4.1a: Evanno's delta  $K$  (Evanno et al. 2005) peaks at  $K = 3$  for  $K = 1-7$  populations. 4.1b: Results of representative STRUCTURE run assuming  $K = 3$  populations. Different colors represent distinct populations.

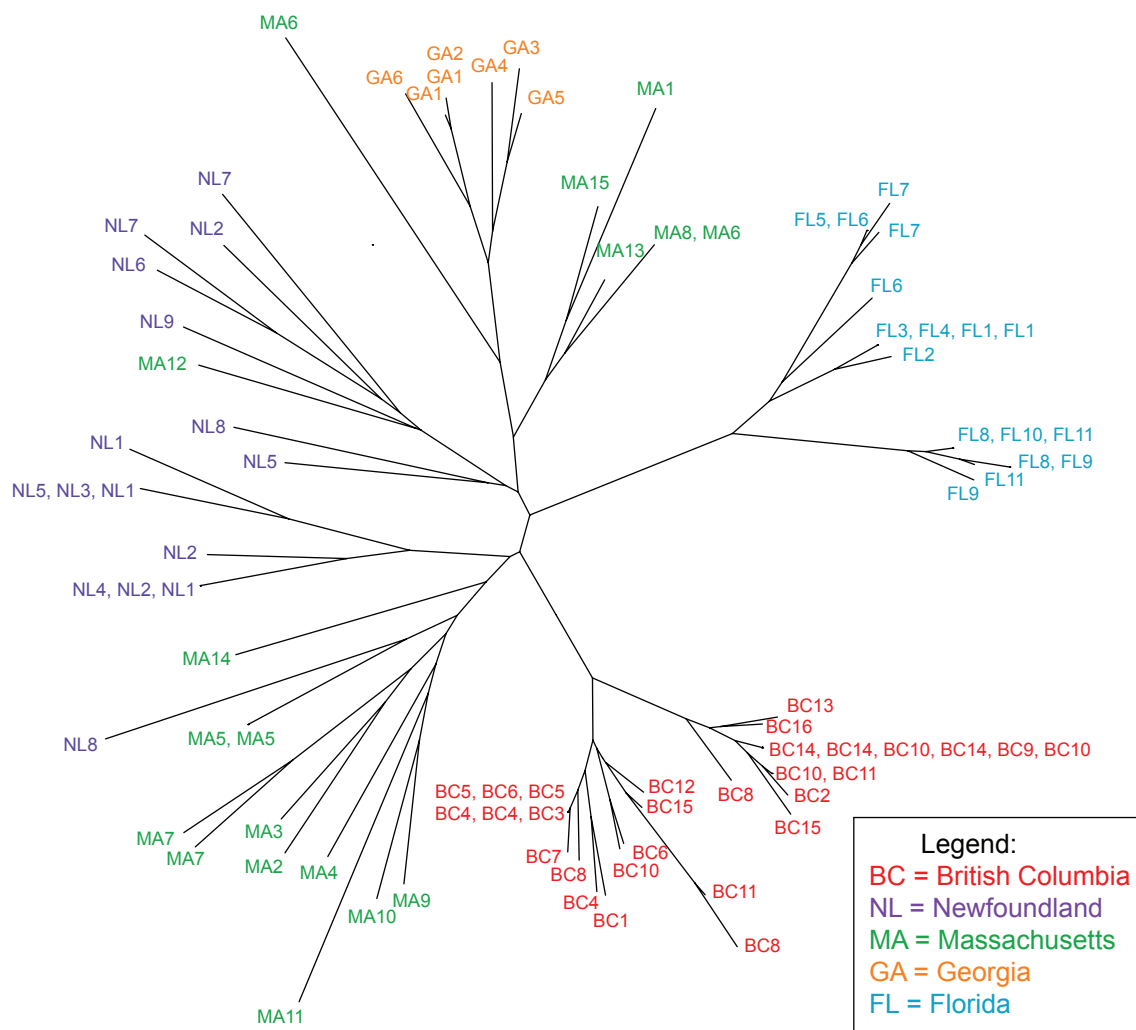


Figure 4.2: Neighbor-joining tree of presence or absence of 72 AFLP peaks in *C. glabrosa* genomes.

British Columbia populations had a higher  $F_{st}$  (0.46) than the eastern population did with either Florida or British Columbia (eastern-British Columbia  $F_{st} = 0.11$ , eastern-Florida  $F_{st} = 0.32$ ). Both STRUCTURE analysis and  $F_{st}$ s produced the same qualitative result when we assumed that *C. glabrosa* is haploid instead of diploid (Figure A3.2, eastern-British Columbia  $F_{st} = 0.39$ , eastern-Florida  $F_{st} = 0.45$ , Florida-British Columbia  $F_{st} = 0.71$ ).

## Discussion

We observed three *C. glabrosa* populations in the five sampled locations: an eastern population (including isolates from Newfoundland, Massachusetts, and Georgia), and Floridian and British Columbian populations. British Columbia was the most remote of the five sampled sites (between ~3900 and ~4600 km from any other sampled site), and housed a distinct *C. glabrosa* population. Florida also housed a distinct population, although it was close to another sampled location (~350 km from Georgia). In contrast, a single population occupied the Newfoundland, Massachusetts, and Georgia sites, covering sites that were 3000 km apart. When we removed all Massachusetts isolates from the analysis, isolates from Newfoundland and Georgia continued to form a single population; we infer that *C. glabrosa* individuals freely mix in this population over at least the distance between Georgia and Newfoundland.

A lack of population structure between Newfoundland and Georgia suggests that *C. glabrosa* is not limited by dispersal on a scale of ~3000 km (the distance between the Newfoundland and Georgia sites). However, limited dispersal coupled with genetic drift most likely caused population divergence between the British Columbian and other



populations. The British Columbia site is at least ~3900 km from any other sampled site, which may be far enough to isolate the British Columbian and eastern populations. We expect further sampling between Newfoundland and British Columbia to reveal either gradual isolation by distance between eastern and western Canada or an allopatric barrier somewhere in Canada.

In contrast to the single large population covering ~3000 km between Newfoundland and Georgia, the Florida *C. glaebose* population, which is only ~350 km from the southernmost eastern site, is distinct from all other populations. There are several hypotheses that may explain division in *C. glaebose* populations over a short spatial scale. Historical factors may shape population divisions; for example, Bermingham & Avise (1986) observed that the Apalachicola basin divides different populations of freshwater fish species (note that our Florida site is located in the Apalachicola basin). They speculated that changes in sea level during the Pleistocene reduced freshwater fish habitat, and that observed population divisions are the result of subsequent contact of populations isolated during the Pleistocene. However, the presence of a large *C. glaebose* population that covers thousands of kilometers suggests that *C. glaebose* is not dispersal limited over hundreds to thousands of kilometers, and therefore we consider it unlikely that such a sea level change would limit *C. glaebose* dispersal, or that isolated *C. glaebose* populations would not quickly mix after contacting one another. For the same reasons, we do not think it is likely that a present-day allopatric barrier limits dispersal between the eastern and Floridian populations.

One or several present-day environmental factors may select different *C. glaebose* genotypes in Florida and Georgia. Over the course of our sampling, we did not measure

fine-scale local environmental conditions; *Candida glabrosa* genotypes may be locally adapted to soil chemistry, pitcher pH, or other environmental factors. We did obtain coarse-scale temperature and precipitation averages for each of the five sampled sites (see Chapter 2). Average yearly temperature differs by 0.1 °C between the Georgia and Florida sites, and we do not consider temperature to be a likely driver of population differences. However, the Florida site has the greatest amount of annual precipitation of all sampled sites, and we consider precipitation a candidate environmental factor that may select *C. glabrosa* genotypes.

Interactions between *C. glabrosa* and other organisms may also shape *C. glabrosa* population structure. We consider selection by *Sarracenia* hosts to be a plausible factor shaping population divergence between Florida and other populations. Despite the proximity of the Georgia and Florida sites, two different *Sarracenia* species occupy the two sites: *S. purpurea* in Georgia and *S. rosea* in Florida. *Sarracenia rosea* is genetically and morphologically divergent from *S. purpurea* (Naczi *et al.* 1999, Godt & Hamrick 1998, Neyland & Merchant 2006, Ellison *et al.* 2004, Ellison *et al.* 2012), and may produce different chemical or physical environments from *S. purpurea* from the point of view of *C. glabrosa* genotypes. Koopman & Carstens (2011) observed a similar pattern with bacteria inhabiting *S. alata* pitchers: different *S. alata* host populations contain different pitcher bacterial communities in Louisiana. Future work to test this hypothesis will sample *C. glabrosa* in more locations in the southern United States, and will test survival of *C. glabrosa* isolates from different locations in *S. purpurea* and *S. rosea* pitchers.

While we expected the same processes to shape fungal communities and *C. glabrosa* populations, different population and community subdivisions do not support this hypothesis. *Candida glabrosa* population structure may correlate with host plant taxonomy, average annual precipitation, or other unmeasured environmental conditions, while fungal community structure correlates with average yearly temperature or a correlated environmental factor. If *C. glabrosa* genotypes continue to correlate with host taxonomy after other *Sarracenia* populations in the southern United States are sampled, it is possible that *C. glabrosa* shares a coevolutionary history with its host plants. *Candida glabrosa* is abundant in pitchers, which may indicate that it is adapted to its host. We further hypothesize that most observed fungal taxa in Chapter 2 are host generalists; the correlation between community composition and temperature, an environmental factor external to pitchers, supports this hypothesis. To test both hypotheses, future work should investigate both population patterns in other pitcher-inhabiting fungal species and physiological relationships among pitcher plants, *C. glabrosa*, and other fungi.

## Conclusions

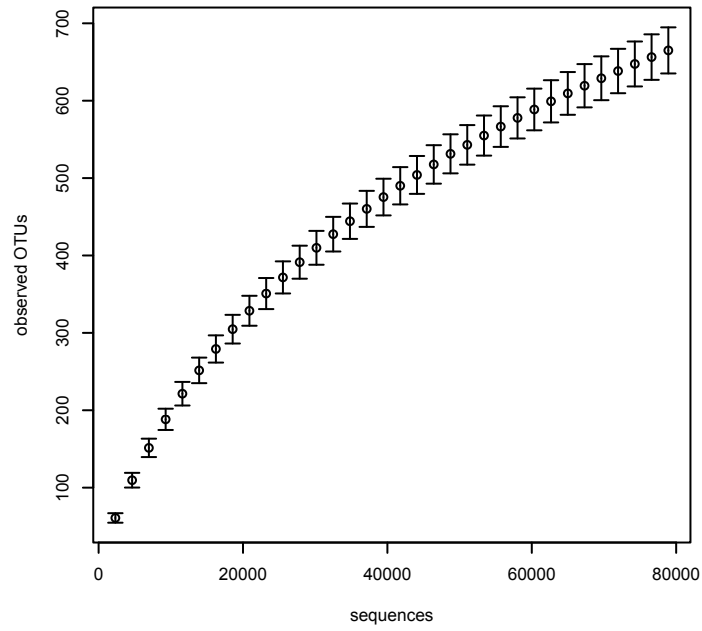
Different ecological processes shape fungal assemblages at community and population scales. *Candida glabrosa* dominated fungal communities, and its population structure contrasted with fungal community structure: *C. glabrosa* population structure correlated with *Sarracenia* taxonomy and, to a lesser extent, geographic distance. Future sampling may reveal that *C. glabrosa* population structure correlates with other environmental factors, including precipitation. In contrast, fungal community composition correlated with temperature and *W. smithii* population structure over space,

and with pH and insect capture rate over time. By describing fungal diversity from both the population and community points of view, we suggest that all three processes (abiotic environmental filtering, interactions with host plant, and interactions with insects) shape fungal diversity in pitchers. Future experimental and observational work will confirm the ecological and evolutionary processes operating at each scale.

## **APPENDIX 1**

### **Supplemental figures for Chapter 2**

A1.1a: OTU accumulation for the geographic data set



A1.1b: OTU accumulation for the temporal data set

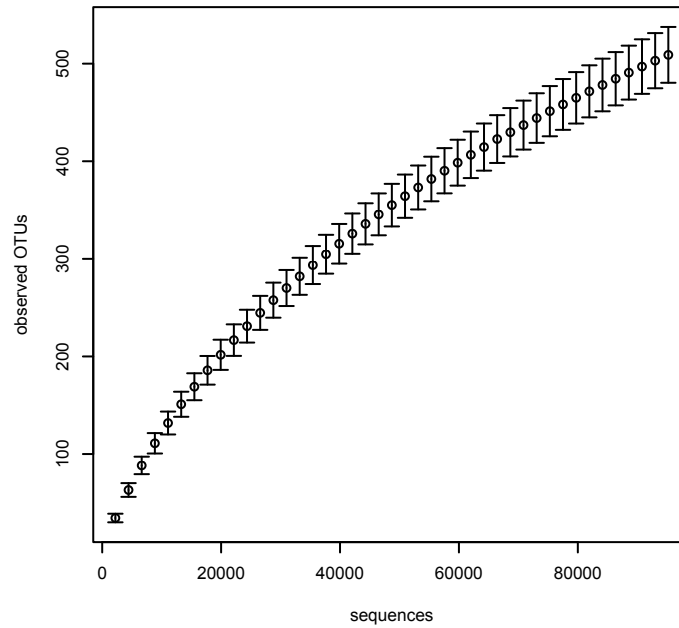
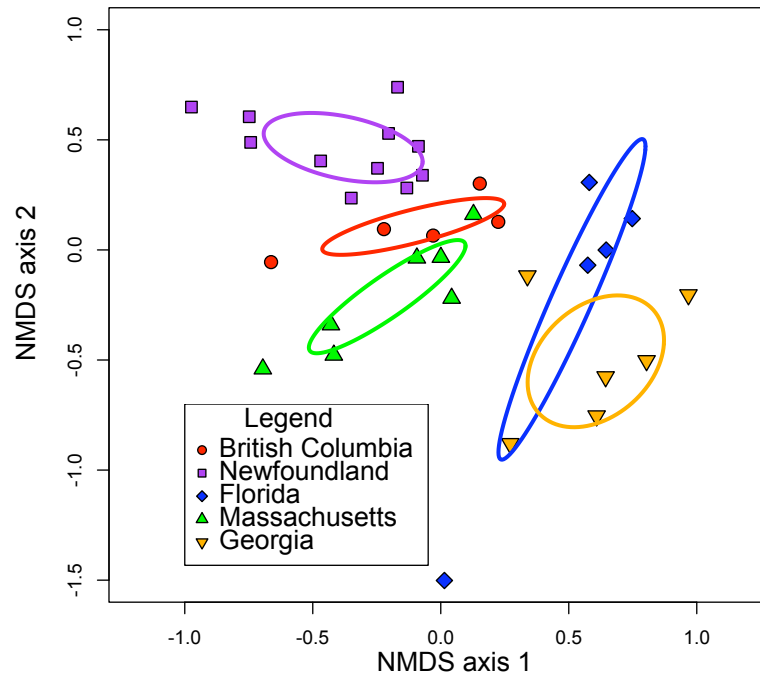


Figure A1.1: OTU accumulation curves for temporal and geographic data sets. The x-axis depicts number of sequences and the y-axis depicts Mau Tau estimates of OTU richness. Bars are 95% confidence intervals. A1.2a: Geographic data set. A1.2b: Temporal data set.

A1.2a: **Fungal taxa by location: Sequence abundance**



A1.2b: **Percentage of sequences in each fungal class by location**

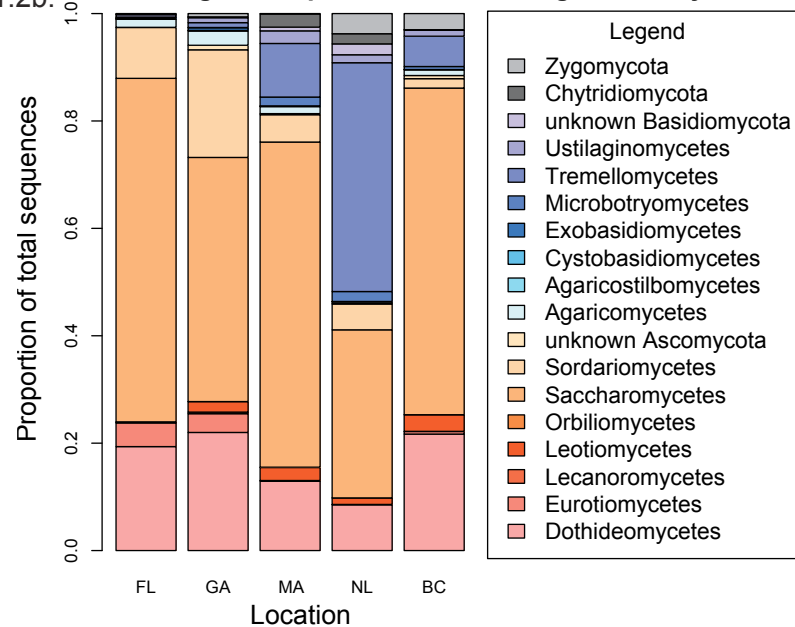
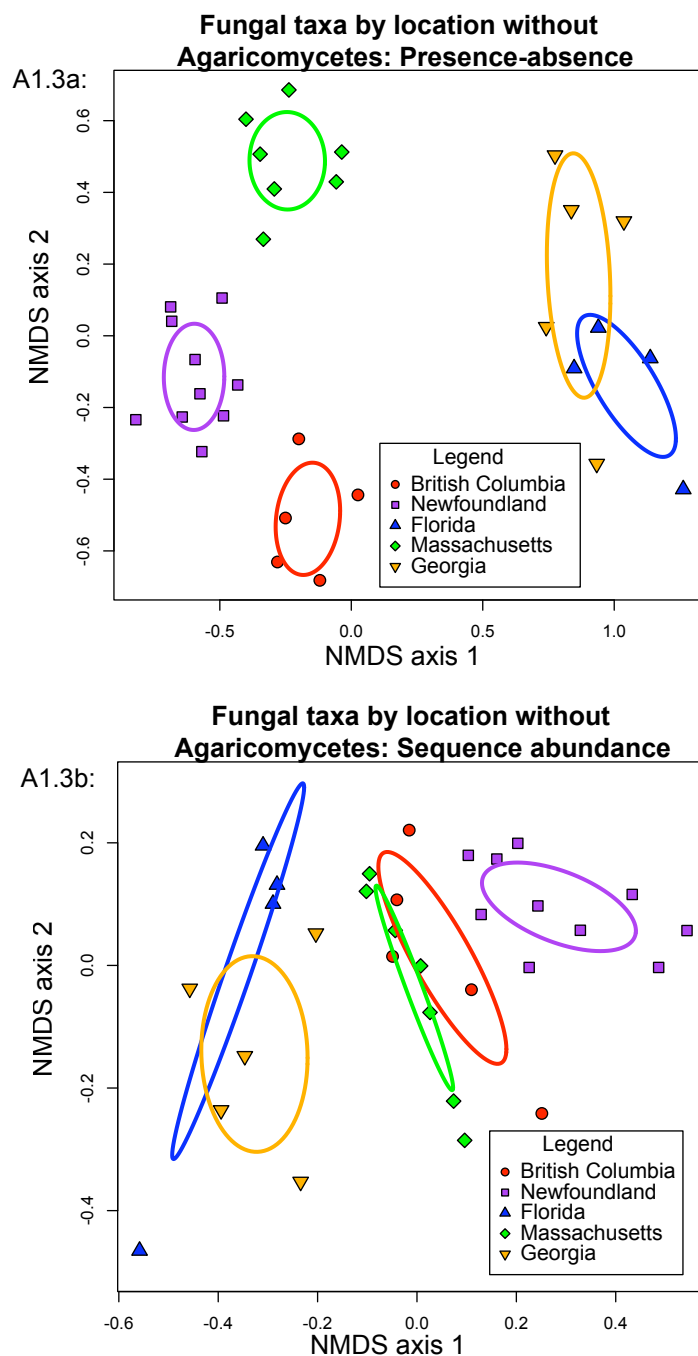


Figure A1.2: Ordination and taxonomy of geographic sequence abundance data. A1.2a: NMDS ordination of transformed geography sequence abundance data. Ellipses depict 95% confidence intervals of centroids of each group. ANOSIM  $R = 0.72$ ,  $p < 0.001$ .

A1.2b: Percentages of sequences in each fungal phylum or class in each location. Ascomycetes are in red and orange; Basidiomycetes are in blue; and basal fungal lineages are in gray.



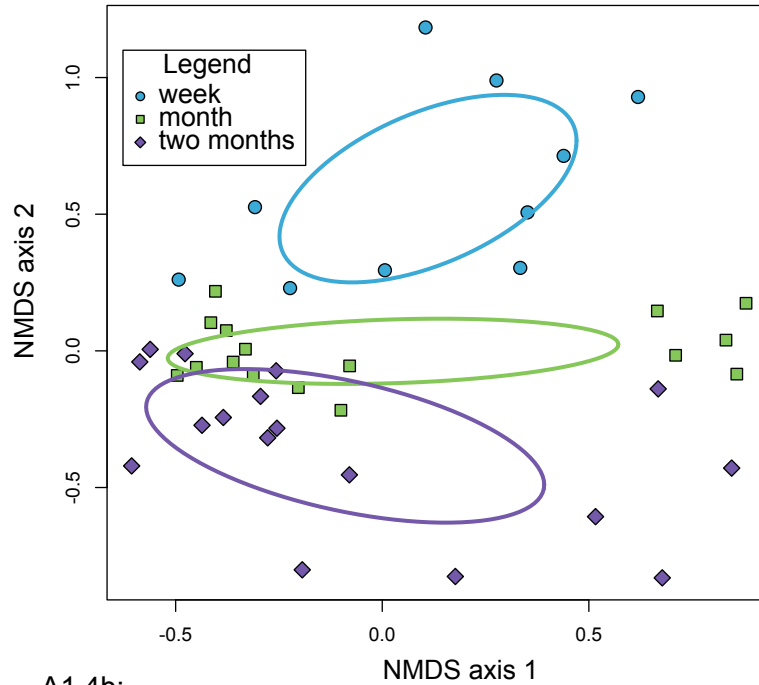
*Figure A1.3: NMDS ordination of geographic data set with Agaricomycetes and unassigned sequences removed.*

*Samples were rarefied to 1033 sequences. Ellipses depict 95% confidence intervals of centroids of each group.*

*A1.3a: NMDS ordination of presence-absence transformed data set. ANOSIM  $R = 0.91$ ,  $p < 0.001$ . A1.3b: NMDS ordination of transformed sequence abundance data set. ANOSIM  $R = 0.74$ ,  $p < 0.001$ .*



A1.4a: Fungal taxa by timepoint: Sequence abundance



A1.4b:

Percentage of sequences in each fungal class by timepoint

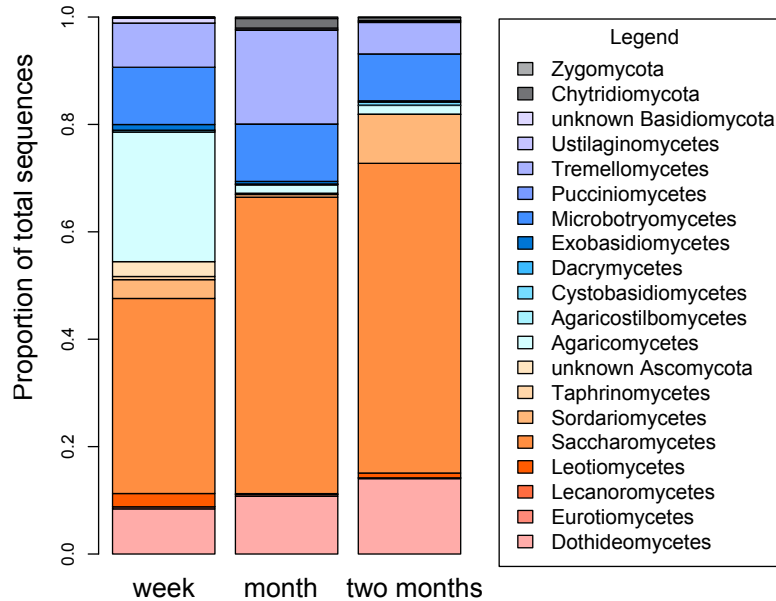


Figure A1.4: Ordination and taxonomy information for temporal sequence abundance data. A1.4a: NMDS ordination. Ellipses depict 95% confidence intervals of centroids of each group. ANOSIM  $R = 0.27$ ,  $p < 0.001$ . A1.4b: Percentages of sequences in each fungal phylum or class in each timepoint. Ascomycetes are in red and orange; Basidiomycetes are in blue; and basal fungal lineages are in gray.

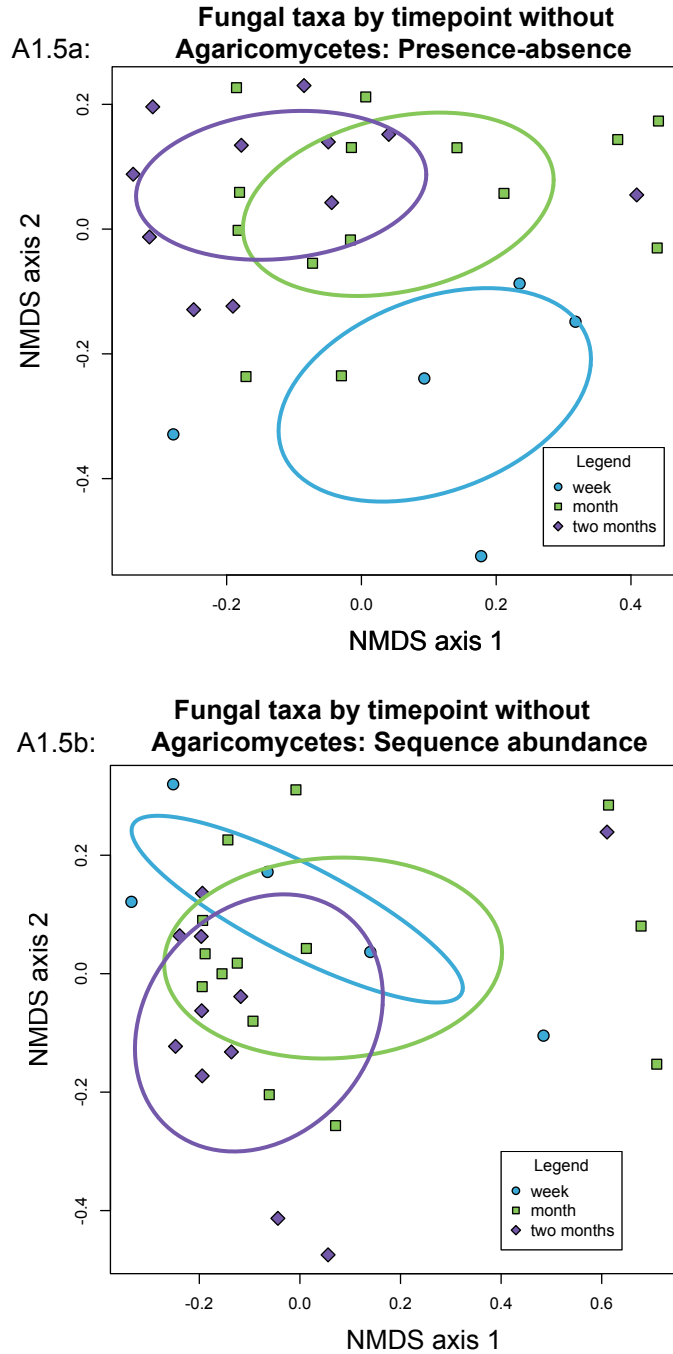


Figure A1.5: NMDS ordination of temporal data set with Agaricomycetes and unassigned sequences removed. Samples were rarefied to 1276 sequences. Ellipses depict 95% confidence intervals of centroids of each group. A1.5a: NMDS ordination of presence-absence transformed data set. ANOSIM  $R = 0.21$ ,  $p = 0.004$ . A1.5b: NMDS ordination of transformed sequence abundance data set. ANOSIM  $R = 0.06$ ,  $p = 0.17$ .

## **APPENDIX 2**

### **Supplemental tables for Chapter 3**

*Table A2.1: Identities of the three yeasts used in this study and the accession numbers of sequences that match ITS and large ribosomal subunit sequences produced from our isolates.*

Yeast	region	matching accession number	query coverage	max identity
<i>Candida glabosa</i>	ITS	FM178351.1	100%	99%
<i>Candida glabosa</i>	Large subunit	FJ432670.1	67%	99%
<i>Rhodotorula glutinis</i>	ITS	AB026018.1	99%	99%
<i>Rhodotorula glutinis</i>	Large subunit	HM627115.1	100%	100%
<i>Pseudozyma aphidis</i>	ITS	HQ662536.1, AB204896.1, AF294699.1	100%	100%
<i>Pseudozyma aphidis</i>	Large subunit	FN424100.1	67%	100%

Table A2.2: Statistics testing for identical slopes and intercepts of two regressions of number of co-inoculated competitor cells on the relative yield of each target yeast (from two-slope *t*-test and ANCOVA, respectively). See Figure 3.3 and Table 3.3.

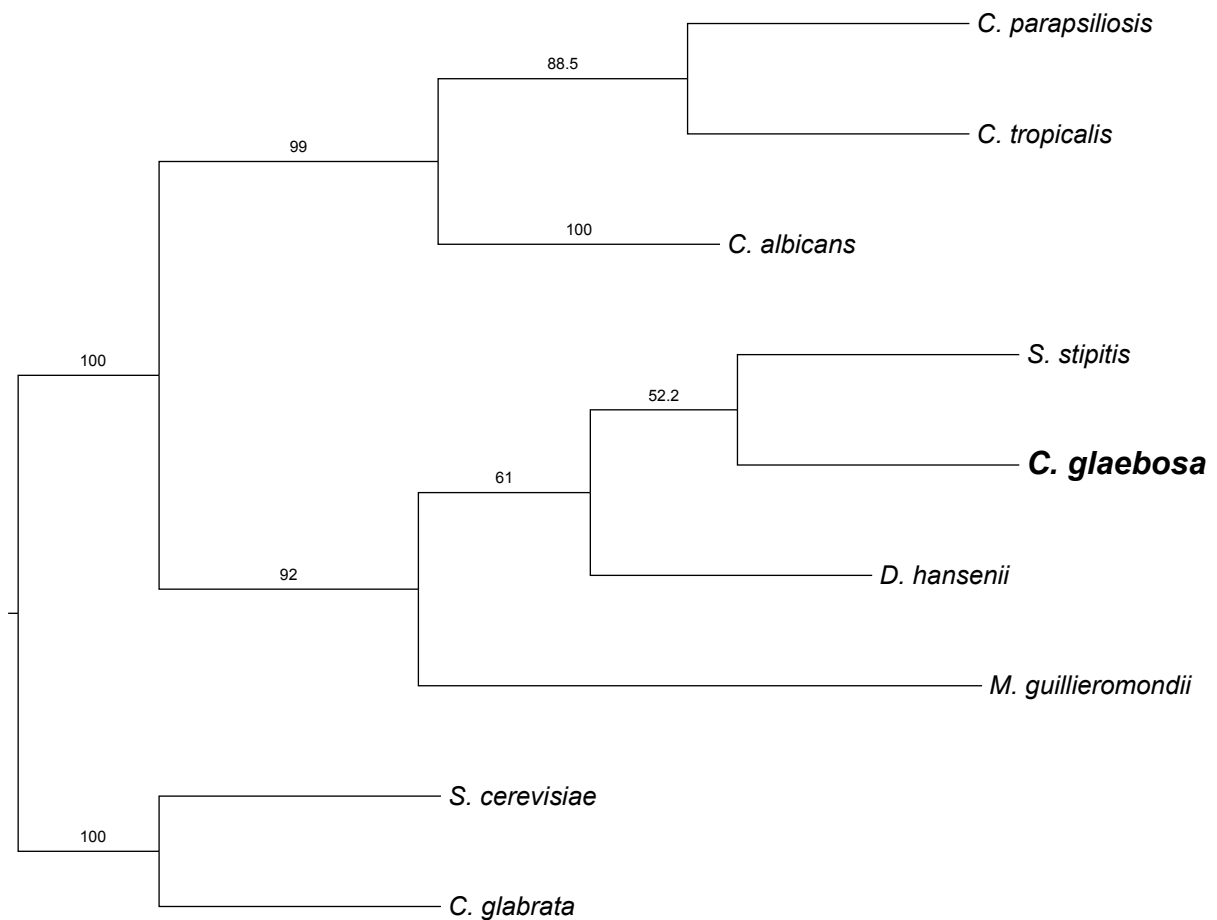
target yeast	slope <i>t</i>	slope <i>df</i>	slope <i>p</i>	competitor × inoculum size <i>t</i>	competitor × inoculum size <i>df</i>	competitor × inoculum size <i>p</i>
<i>C. glabrosa</i>	-2.67	40	0.01	--	--	--
<i>R. glutinis</i>	3.6	48	$7.6 \times 10^{-4}$	--	--	--
<i>P. aphidis</i>	0.57	46	0.57	-0.552	46	0.58

Table A2.3: Mantel test statistics of the presence of each yeast in a pitcher correlated with distance between pitchers.

Yeast	Time	Mantel $r$	$p$
<i>C. glabrosa</i>	4 days	0.15	0.42
<i>C. glabrosa</i>	1 week	-0.04	0.81
<i>C. glabrosa</i>	1 month	-0.05	0.86
<i>C. glabrosa</i>	2 months	0.07	0.07
<i>R. glutinis</i>	1 week	-0.09	0.93
<i>R. glutinis</i>	1 month	-0.10	0.99
<i>R. glutinis</i>	2 months	-0.03	0.70
<i>P. aphidis</i>	2 months	0.01	0.44
Fungal ITS	4 days	$1.61 \times 10^{-3}$	0.43
Fungal ITS	1 week	-0.04	0.73
Fungal ITS	1 month	-0.01	0.52
Fungal ITS	2 months	$3.77 \times 10^{-5}$	0.47

## **APPENDIX 3**

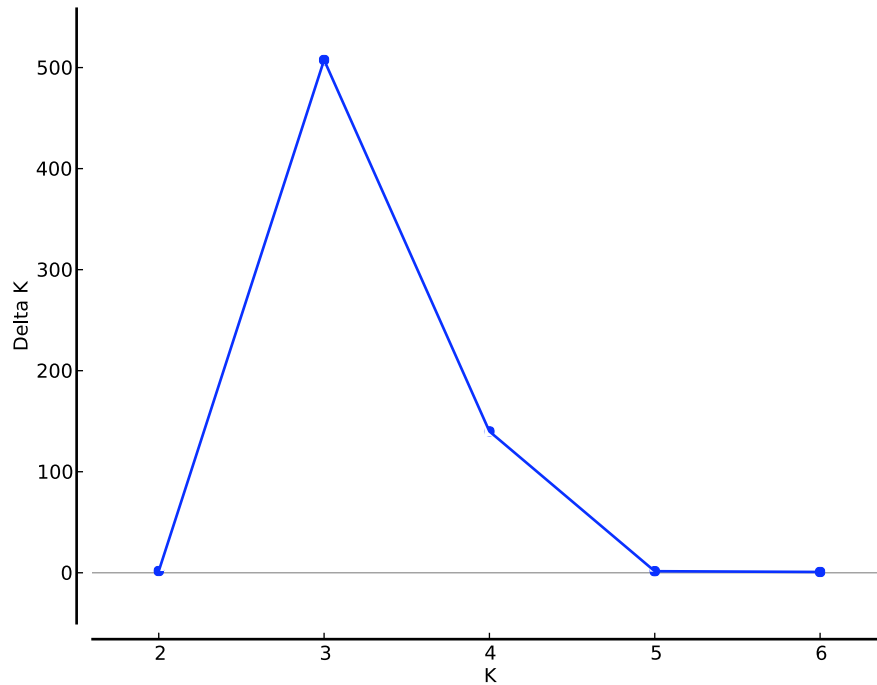
### **Supplemental figures for Chapter 4**



*Figure A3.1: Preliminary consensus bootstrap maximum parsimony tree of the D1/D2 ribosomal region of one Massachusetts Candida glabrosa isolate and eight Ascomycete yeasts. Yeast sequences are taken from the NCBI database, and have the following accession numbers: Scheffersomyces stipitis JQ025391.1, Saccharomyces cerevisiae JN938921.1, Candida albicans JN940614.1, Candida glabrata JN940615.1, Debaryomyces hansenii JN938932.1, Meyerozyma guilliermondii JN940622.1, Candida parapsilosis JN940630.1, Candida tropicalis JN940626.1. Phylogenetic tree created using Phylip version 3.69 (Felsenstein 1993).*



A3.2a:



A3.2b:

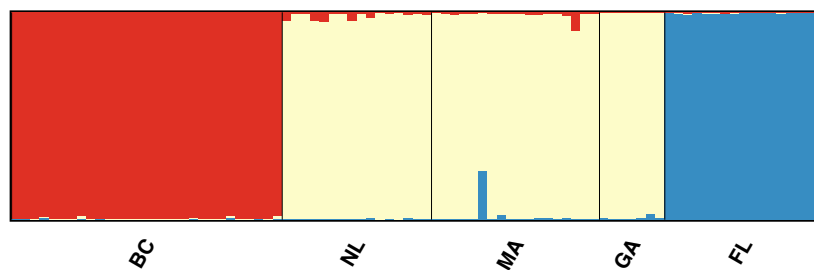


Figure A3.2: *Candida glabrosa* populations predicted by *STRUCTURE*, assuming haploidy. A3.1a: Evanno's delta  $K$  (Evanno et al. 2005) peaks at  $K = 3$  for  $K = 1-7$  populations. A3.1b: Results of representative *STRUCTURE* run assuming  $K = 3$  populations. Different colors represent distinct populations.

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